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PREFACE

In introducing the present volume of the *Annual Review of Biochemistry* to its readers, the Editors and the Editorial Committee feel that a word of explanation is called for concerning the fate of the section hitherto devoted to plant biochemistry. The present policy of the Directors favors transfer to the *Annual Review of Plant Physiology* of a few subjects which may reasonably be considered to fall quite clearly within the domain of plant biochemistry, plant physiology, and plant nutrition, as, for example, Mineral Nutrition of Plants, Growth Substances in Plants, and Plant Respiration. There are, however, other subjects which are more borderline in character, which interpenetrate the broad field of general biochemistry, and which are of obvious interest to biochemists at large, such, for example, as Photosynthesis and Plant and Animal Pigments. These borderline topics will be considered year by year by both Editorial Committees with the thought in mind of continuing their inclusion in the *Annual Review of Biochemistry*, possibly by a system of alternation as between the two *Reviews*, by reprinting, or by some other mutually acceptable plan.

Much of the biochemical research pursued on plants will continue to be reviewed in the *Annual Review of Biochemistry*, although less conspicuously than the topics alone would indicate. This follows from the very nature of biochemical research: enzyme preparations for use in fundamental studies of wide interest are derived from plants, animals, and microorganisms alike; the chemist whose research is devoted to carbohydrates, to fats, or to the virus proteins may turn to the most diverse organisms, plant or animal, in seeking out the materials to be studied; and certainly the many who are interested in animal and human nutrition are constantly obliged to turn to plants for many of the foodstuffs and accessory organic nutrients upon which we depend. The entire fabric of biochemistry is woven from all parts of the living world.

To all of those who have participated in the preparation of the present volume, we extend our thanks. The task of the authors is becoming no easier as the subject itself expands and new journals of original publication are born overnight. We encourage the author to avoid any attempt to review most of the papers embraced by the topic of his review. He is urged to select only a limited number, those which are of outstanding significance, and from these to prepare a critical appraisal of the present status of the subject. The difficulties in achieving this end are self-evident, but when accomplished, the resulting review is almost invariably a source of satisfaction to the Committee and of great value to the reader.

To our editorial assistants, whose knowledge of the language of biochemistry is fast becoming encyclopedic, we express our sincere appreciation for their splendid help. To our printers, the George Banta Publishing Co., we are likewise indebted for their fine co-operation.

H.J.A. H.S.L.
A.K.B. J.M.L.
H.J.D. G.M.
H.A.S.

DENNIS ROBERT HOAGLAND
1884–1949

Dennis Robert Hoagland, Professor of Plant Nutrition at the University of California, died on September 5, 1949. It is not our purpose in these few paragraphs to outline his illustrious contributions to plant physiology, to recall the many honors that were bestowed upon him, or to describe the fine personal qualities of Hoagland, the man. Much of this has appeared elsewhere [*Plant Physiology*, 25, V-XVI (1950)]. It is sufficient, perhaps, if we mention something of Hoagland's relationship to the *Annual Review of Biochemistry* and the many years of close association that some of us enjoyed with him in connection with this *Review*. It was in July, 1930 that the initiation of such a series was first proposed to Hoagland. He responded with characteristic enthusiasm and wrote for Volumes I and II the introductory reviews on Mineral Nutrition of Plants. From the beginning, he served as a member of the Editorial Committee and of the Board of Directors. In the former capacity, Hoagland's assistance was remarkable. His knowledge of the research in soil science and plant biochemistry currently in progress throughout the world was amazing—a reflection of his own absorbing interest in his science. Hoagland's knowledge of the men engaged in such research was equally broad. In consequence, he brought to the meetings of the Committee a fund of information that could hardly be surpassed.

Hoagland was always eager that the *Reviews* be maintained at a fundamental level—as close to the basic sciences as possible. Even in his own field, and while fully appreciative of the great body of valuable research in progress in agriculture and the applied phases of plant physiology, he was ever insistent that topics of an immediate, practical interest be excluded from the *Review*. For similar reasons, he favored exclusion of chapters on clinical and pathological chemistry. As early as September 1, 1930, in his first letter concerning the enterprise, he virtually prophesied the establishment of an *Annual Review of Medicine:* "If desired, a separate volume should be issued to deal with the medical side, in the sense I have in mind."

Hoagland had long been in favor of establishing an "Annual Review of Plant Physiology," or an "Annual Review of Plant Physiology and Plant Nutrition," as he would have preferred to designate it. The correspondence on this goes back to 1936. In 1942 he wrote, "Some day I hope that such a Review may be initiated, but obviously this is not the time." In 1945, as soon as the war ended, the necessary exploratory steps were taken for the publishing of an "Annual Review of Soil Science and Plant Physiology." By January, 1948, the Board of Directors was ready to approve the project. Hoagland's interest in this new *Review* was by then much to the fore, and his fellow members of the Board profited immeasurably from his advice and sound judgment. Though physically incapacitated for the last three years of his life, his mind was remarkably keen. He rarely attended meetings during

this period and wrote comparatively few letters as his infirmities grew upon him. In one of these letters, he summarized his concept of the scope and functions of the new *Review*:

"If we begin an Annual Review of Plant Physiology it should be on a broad scope to include Plant Nutrition . . . I do not have in mind, of course, a Review that will cover the practical empirical phases of plant nutrition, but simply the purely scientific aspects of it."

It was a source of tremendous satisfaction to Hoagland to participate in the execution of the plan, which had long been discussed, of publishing an annual review devoted to the fundamentals of the science to which he had devoted his life and to know that the new undertaking was in hands in which he had complete confidence.

J.M.L.
H.A.S.

TOPICS AND AUTHORS

ANNUAL REVIEW OF BIOCHEMISTRY

VOLUME XX (1951)

ERRATA

Volume XVIII

page 548, line 33: *for* antiserine *read* antithreonine.

CONTENTS

BIOLOGICAL OXIDATIONS[1]

By Van R. Potter

McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wisconsin

Krebs Oxidative Cycle

The citric acid cycle, first outlined by Krebs some 12 years ago, continues to serve admirably as a means of integrating a vast amount of data in the field of biological oxidations. Stern & Ochoa (1) demonstrated that citric acid was formed in the presence of an enzyme preparation supplemented with acetate, oxaloacetate, coenzyme A, adenosinetriphosphate (ATP) and magnesium ions. The preparation contained almost no aconitase on the basis of tests in which *cis*-aconitate or isocitrate was added and very little citrate found. It was therefore concluded that citrate is the product of the Krebs condensation [see also (2)] and that oxaloacetate and acetate are the substrates involved. This experiment also established the participation of coenzyme A in the reaction [see also (3)] and showed that pyruvic acid is probably oxidized to an acetate rather than participating *per se* in the condensation reaction.

Ogston (4) pointed out that the two primary carboxyl groups in citric acid are not equivalent in that it can not be concluded that the two groups will react similarly in an enzyme-substrate complex, in which the citrate molecule would presumably be oriented. By isolating citrate from a homogenate in which isotopic carbon dioxide had been incorporated into the intermediates of the Krebs cycle, Potter & Heidelberger (5) were able to show that the citrate was indeed asymmetrical. When the isolated citrate was enzymatically oxidized to α-ketoglutarate and the latter was oxidized to succinate and carbon dioxide, the latter contained essentially all the isotope from the α-ketoglutarate, while no isotope was found in succinate. On the other hand labeled succinate was obtained from the urine of rats that had received labeled acetate (6) and experiments with yeast also indicated that labeled acetate enters that portion of the citrate molecule from which succinate is derived (7). In the latter experiments the recycling of the labeled succinate led to the formation of citrate from labeled C_4 and C_2 fragments, with the result that the isotope appeared in both the primary and the tertiary carboxyl groups. The available data indicate that both the synthesis and the degradation of citrate proceeds on an asymmetric basis, so that entering C_4 carboxyls become carbon dioxide and the entering C_2 carboxyls become C_4 carboxyls.

Studies on the ability of various tissues to fix nonlabeled carbon dioxide showed that pigeon liver was almost 20-fold more active than most other tissues examined (8).

[1] This review covers approximately the period from November 1948 to November 1949 but it must not be regarded as a complete survey.

There have been a number of publications based upon the use of fortified homogenates or preparations derived therefrom for the study of Krebs cycle oxidations. The original work by Krebs and his associates was done with minced tissue preparations [and not with homogenates as was recently stated (9)]. In minced preparations of tissues, such as pigeon breast muscle, vigorous oxidation could be observed without the addition of cofactors. Later work with homogenates has been made possible largely by the addition of ATP to the reaction mixture, although the elimination of "destructive" enzymes by centrifugation of the active material (9) and the precautions of speed and cold are also helpful. During the last year further experiments with whole homogenates have been carried out in the author's laboratory (10 to 14), while extensive studies with washed particles have been continued in the laboratories of Green in the cyclophorase series (15 to 20), of Hunter (21, 22, 23), and of Lehninger (24 to 27) but the nature of the enzyme system that carries out the Krebs cycle oxidations and related phosphorylations is still far from being understood.

It has become apparent that the bulk of the oxidative system is in the large granules of the cytoplasm (mitochondria). Schneider & Potter (28) found that the mitochondria had 30 to 50 per cent of the oxidative activity of whole homogenates. The remaining cell fractions were nearly inactive when tested alone but were able to increase the activity of the mitochondrial fraction. Kennedy & Lehninger (25) obtained similar results with the isolated fractions but did not compare mitochondria with whole homogenates or recombined fractions.

The nature of the oxidative enzyme complex that is associated with the mitochondria has been attacked chiefly by Green and by Lehninger, and their coworkers, although data from these and other laboratories also have a bearing on the conclusions to be drawn.

The Green school began by studying the "cyclophorase" preparation in terms of the metabolic pathways followed by carbohydrate and fat (papers I–IV)[2]. During the current year these studies were extended to include proline and hydroxyproline (15) and D-aspartic acid (17), but in the latter instance, the enzyme could readily be spilt off from the particles. The first real indications of the nature of the cyclophorase concept[2] appeared in Paper VI (16), of the series which concerned oxidative phosphorylation. It was shown that during the oxidative reaction, inorganic pyrophosphate was formed from orthophosphate when no other phosphate acceptor was present. In order to transmit phosphate to acceptor systems it was necessary to add a soluble transphosphorylase such as yeast hexokinase to the washed

[2] For the purposes of this review it seems desirable to distinguish between the cyclophorase preparation (a washed residue obtained by centrifuging the product procured from a Waring blendor), the cyclophorase system of enzymes (the enzymes that catalyze the Krebs oxidative cycle), and the cyclophorase concept (a mosaic of organized enzymes with nondissociating coenzymes). Papers I–IV refer to the work by Green and co-workers on the cyclophorase system published in the J. Biol. Chem

particle preparation. In discussing the adenylic acid requirement of the system it was stated that this compound served in a two-fold capacity; it was thought to combine with the apo-oxidase to restore the activity of the various oxidative systems (referred to as "adenyloproteins") and secondly it served as a catalytic phosphate-transmitting system. It was stated that the preparation contained large amounts of bound coenzymes including coenzymes I and II, coenzyme A, flavin adenine dinucleotide and diphosphothiamine. It was proposed that phosphorylation is coupled to oxidation by the formation of reduced coenzyme pyrophosphates.

Studies with radioactive phosphorus (18, 19, 20) showed that cyclophorase contains a very labile form of phosphate ("Gel P") that is not distinguishable from inorganic phosphate analytically. This phosphorus was said to remain in the residue after several washings in large volumes of cold media at the centrifuge, but was released from the gel by trichloroacetic acid, and determined as inorganic phosphate. In addition, significant amounts of P^{32} were present as ATP or adenosinediphosphate (ADP), and as an adenine polyphosphate not identical with either of these two compounds (19). Both the absolute amount of "gel P" and the specific activity in terms of P^{32} were shown to be related to the occurrence of oxidative processes, and various procedures that result in the loss of oxidative activity were shown to result in the loss of previously incorporated P^{32}, as if some labile coenzyme-phosphate linkage had been disrupted.

Further data bearing on this point have been published by Hummel & Lindberg (29) who prepared whole homogenates of rabbit liver, aerated in the presence of P^{32}-labeled orthophosphate, and determined the P^{32} content of various nucleotide fractions obtained from the reaction mixture. The flavin mononucleotide, pyridine nucleotides and adenylic acid fractions contained no appreciable radioactivity. Only those fractions corresponding to ATP and flavin-adenine dinucleotide were uniformly active. The latter compound was therefore concluded to be intimately associated with aerobic phosphorylation. The pyridine nucleotides are not necessarily excluded as phosphate transmitters by these data if the coenzymes transmit phosphate by forming pyrophosphate linkages when they are in the bound form, as suggested by Green *et al.* (16) since the coenzymes isolated may well be at a lower level of phosphorylation than the coenzymes attain in the tissue.

There is a strong possibility that the adenine nucleotides represent soluble, dissociable phosphate transmitters that maintain phosphorylative connections between the numerous phosphate donors and acceptors in the cell; when cells are homogenized and the contents are diluted these connections are lost unless the molarity of the adenine nucleotides is maintained in the solution as a whole, in contrast to the fact that the hydrogen-transmitting coenzymes are not lost from the enzymes of the Krebs cycle in isotonic homogenates and in certain preparations derived therefrom. The adenine nucleotides may also have the additional effects of either preventing the cleavage of the bound coenzymes from their proteins or of restoring the holoenzyme by transphosphorylation. Aside from the uncertainty as to the vari-

ous functions of ATP in these preparations, the cyclophorase system was pictured as a complex of holoenzymes from which the coenzymes do not readily dissociate, and it was shown that the properties of this system differ considerably from the classical dehydrogenases that are analogous to it (16). The probable localization of the cyclophorase system in mitochondria was referred to earlier. More recently it has been proposed that the classical dehydrogenases are formed from the holodehydrogenases by the loss of the bound coenzyme and the solubilizing of the tissue particles (Green & Huennekins, unpublished). It was reported (16) that supplementation by coenzymes was not necessary in the system provided that adequate amounts of adenylic acid or ATP were present. These observations are paralleled by studies on whole homogenates (10, 14). The studies on cyclophorase may be unnecessarily complicated by the use of a Blendor which disintegrates subcellular structures much more than do the Potter-Elvehjem homogenizers that have been employed in most of the other studies reported in this section. In a comparative study (12) nuclear fragments were found in the supernatant from a cyclophorase preparation, while they are never found in the supernatants from homogenates as prepared in this laboratory. In this connection it may be mentioned that nuclear disintegration may occur with glass homogenizers that are so tight as to produce glass powder during the homogenization.

Friedkin & Lehninger (24) reported the uptake of P^{32} into both acid soluble and acid insoluble fractions in connection with the oxidation of malate. Later studies with reduced diphosphopyridine nucleotide ($DPN \cdot H_2$) indicated that phosphorus uptake was coupled with the oxidation of this compound (26) as had been long suspected. The use of $DPN \cdot H_2$ was, however, discarded in favor of the β-hydroxybutyrate oxidizing system (27). In the latter case, an absolute diphosphopyridine nucleotide (DPN) requirement could be demonstrated by short incubations of the washed enzyme preparation at 30°. Lehninger suggested that the phosphate uptake occurred with the oxidation of $DPN \cdot H_2$ and not with the oxidation of the primary substrate in this system, but it is experimentally difficult to prove that the oxidation of $DPN \cdot H_2$ is the sole source of phosphate uptake in these experiments.

Hunter (21) carried out studies on a similar enzyme preparation in which the oxidation of α-ketoglutarate to succinate was coupled to the reduction of oxaloacetate or acetoacetate. Approximately one mole of phosphate was taken up per mole of substrate oxidized. This figure is quite close to that obtained by Cross *et al.* (16). Hunter & Hixon found that one mole of phosphate was taken up in the anaerobic dismutation of α-ketoglutarate to succinate and glutamate in the presence of ammonia (22) while four moles of phosphate were taken up per atom of oxygen in the oxidation of α-ketoglutarate to succinate (23) which would correspond to one mole per substrate molecule oxidized and one for each of three stages of hydrogen transfer. The phosphorus:oxygen ratio for pyruvate, isocitrate, malate and succinate oxidation were inferred to be 4, 3, 3, and 2 respectively. Although tempera-

tures lower than 38°C. have been previously employed in this type of work, this study emphasized the point, and the experiments were done at 15°C. to minimize the occurrence of phosphate "leaks."

In studies on whole homogenates it was shown that oxaloacetate could be rapidly oxidized with a temporary accumulation of citrate in liver and kidney, while in heart or brain homogenates no citrate accumulated (10). However, in the latter, the maximum rate of oxygen uptake was not attained unless pyruvate was added in addition to oxaloacetate, indicating that the decarboxylation of oxaloacetate was rate-limiting in these tissues. In Flexner-Jobling and Walker 256 rat tumors, the oxygen uptake and citrate formation were both negligible (10). Potter & LePage (11) pointed out that the ability to metabolize oxaloacetate was dependent upon the maintenance of ATP, and they therefore tested the tumors in a glycolytic system in which ATP could be maintained, and showed that a significant amount of oxaloacetate disappeared in addition to what was recovered as pyruvate, lactate and malate. However, the citrate formation was negligible. The available evidence suggests that the Krebs condensation requires the maintenance of high energy phosphate in the system, and that unless this maintenance is demonstrated it cannot be concluded that the oxidative system has functioned at its maximal rate. It is thus difficult to assess the meaning of data on pyruvate oxidation by brain homogenates that were prepared in water, in which a DPN requirement was demonstrated (30). Pyruvate was burned incompletely and the authors were unable to reconcile their results with those of Green *et al.* (9) but at present it appears possible that the system used by Larner *et al.* (30) was disrupted to the point that the phosphate balance could not be maintained.

Fluoroacetate studies.—Kalnitsky (31) reported on the Krebs condensation as observed in homogenates of rabbit kidney and in fractions thereof. The citrate-forming system was concentrated almost exclusively in the mitochondria fraction, in agreement with the results on the oxaloacetate oxidizing system (12) which involves essentially the same enzymes. In the presence of fluoroacetate the yield of citrate was approximately doubled. The system gave better results in oxygen than in nitrogen although in the presence of excess oxaloacetate no oxygen would be needed for the reaction *per se.* Although the phosphate balance was not measured, it is likely that in oxygen there was a higher level of phosphate uptake than in nitrogen. Studies on acetoacetate metabolism also showed a much higher yield of citrate in the presence of oxygen (32). This report is noteworthy for the elegant balance sheets drawn up for components of the Krebs cycle.

The mechanism of fluoroacetate action has been reinvestigated by Peters. The increased citrate formation produced by fluoroacetate, which seemed paradoxical if fluoroacetate inhibited acetate metabolism (Annual Review of Biochemistry, Vol. XVIII, p. 3) was explained on the basis that fluoroacetate does not inhibit the acetate-metabolizing enzyme but that it is actually indistinguishable from acetate in these reactions (33). It was proposed that fluoroacetate condensed with oxaloacetate to give fluorocitrate which

was either toxic *per se* or converted to a product that inhibited an enzyme necessary for citrate oxidation, thus causing citrate to accumulate. Experiments by Martius (34) included fluorosuccinate and fluorofumarate which were not inhibitory and it seems possible that the enzyme inhibited is either aconitase or isocitric dehydrogenase. The *in vitro* experiments were extended to whole animals by Buffa & Peters and it was shown that very great increases in citrate occurred in the brain, heart, and kidneys of rats in 1 hr. following the injection of 5 mg. of fluoroacetate per kg. body weight (35). These results have been confirmed by Potter & Busch (36), who extended the data to include a large number of other tissues, including the tumors previously studied by the homogenate technique (10, 11). Citrate production was low or negligible in liver, testis and tumor tissues. It now appears that fluoroacetate offers the possibility of studying the Krebs condensation specifically by blocking the reactions subsequent to it, provided the active inhibitor is formed, while the identification of the latter should make it possible to carry out more direct experiments.

Endergonic reactions.—The operation of the Krebs cycle as a means of maintaining phosphate bond energy has proved helpful in carrying out various endergonic reactions, some of which have also been carried out in simpler systems that were incapable of respiring but were capable of utilizing ATP. A classical example of the two types of systems has to do with the conversion of citrulline to arginine, which was studied by Ratner & Pappas. They carried out the reaction both in liver homogenates (37) and in a nonrespiring system (38). Since the endergonic syntheses involve oxidative enzymes only indirectly, they will not be discussed further here, but will be found in other chapters. Studies on the accumulation of glutamate in tissue slices showed that the permeability of brain tissue for glutamate is controlled by energy-giving metabolic processes (39).

Plants and microorganisms.—Laties (40, 41) reported data to show that in excised barley roots and in spinach leaves, pyruvate is oxidized to succinate in the presence of malonate, suggesting the occurrence of the Krebs cycle. Pucher & Vickery (42) drew similar conclusions from studies on detached tobacco leaves. Conn *et al.* (43) observed oxidative decarboxylation of malic acid by a TPN-specific enzyme in tissue from a variety of plants. Conversion of pyruvate to fumarate via the Wood-Werkman reaction was reported to be inhibited by high tensions of carbon dioxide in the mold *Rhizopus nigricans* (44) and the results were related to the inhibition of growth under similar conditions.

Ajl & Werkman (45, 46) have studied the mechanism of growth stimulation by carbon dioxide in bacteria, and report that the C_5 acids replace carbon dioxide to a greater extent than the C_4 acids of the Krebs cycle, indicating a possible carbon dioxide fixation in the back reaction of succinate to α-ketoglutarate. Direct evidence of this reaction was also reported (47).

Smith (48) proposed that oxalate was formed from acetate via glycolate and glyoxalate in the molds *Merulius lacrymans* and *Marasumus chordalis*,

while Lynen & Lynen (49) proposed a modified Krebs cycle in *Aspergillus*.

Formation of different ratios of acetate:butyrate from fumarate and malate in *Clostridium saccharobutyricum* led to the postulation that malate was not an intermediate in the conversion of fumarate to oxaloacetate in these experiments (50).

An outstanding series of papers by Stadtman & Barker (51 to 55) reported work with cell-free enzyme preparations obtained from *Clostridium kluyveri*. The metabolic pathway studied does not proceed via the Krebs cycle, but the methods and findings represent a great contribution to the same basic issues dealt with by other reports in this section. The authors were concerned with the intermediates in the conversion of ethanol to butyric acid, and they succeeded in obtaining extracts that would convert ethanol to butyric acid by two dehydrogenations and two hydrogenations via acetaldehyde, acetyl phosphate, and two C_4 compounds not identical with acetoacetic acid or other common C_4 acids. Acetoacetate yielded acetate and acetyl phosphate in a phosphoroclastic reaction. The individual hydrogenations and dehydrogenations could be carried out with hydrogen and oxygen uptake respectively. Acetoacetyl phosphate might be considered as a possible C_4 intermediate but no data on this substance are available.

Dehydrogenases

Succinic dehydrogenase.—It has been widely realized that the succinic oxidase system consists of more than succinic dehydrogenase, cytochrome-*c*, and cytochrome oxidase, but the complexities of this relatively simple enzyme system continue to challenge investigation. Keilin & Hartree (56) rejected all previous claims regarding additional components except data by Slater whose studies led him to propose the existence of a new catalyst between cytochrome-*b* and *c* (57 to 61). Methylene blue was considered to interact as an oxidant of cytochrome-*b* in the succinic system (58 cf. 62), and it was suggested that unknown hematin compounds with spectra not normally visible occurred in the preparations. Experiments with British anti-Lewisite (BAL) were explained on the basis that a hematin compound necessary for the transmission of electrons between cytochromes-*b* and *c* was destroyed (60). The same factor was shown to act between diaphorase, which oxidizes reduced cozymase, and cytochrome-*c*, in the absence of cytochrome-*b*. (57). New techniques of low temperature spectrophotometry (63) led to the discovery of a new cytochrome, called *e*, which is not visible at ordinary temperatures. Whether this component is identical with the Slater factor remains to be seen. Slater (61) also studied the role of the SH group in succinic dehydrogenase and reported that the shielding phenomena (protection against sulfhydryl inhibitors by succinate and malonate) might have to be reinterpreted on the basis of evidence that the sulfhydryl inhibitors acted elsewhere than on the dehydrogenase.

On the basis of studies with extracts from diphtheria bacilli and from beef heart muscle cytochrome-*b* and succinic dehydrogenase were considered

as possibly identical by Pappenheimer and Hendee (62). A possibly similar preparation from an endotoxin from *Salmonella aertrycke* also oxidized succinate aerobically, contained no cytochrome oxidase, but was capable of reducing cytochrome-*c* (64). Ball & Cooper (65) studied the stability of succinoxidase and concluded that decomposition was caused by a phosphatase on the basis of stabilizing effects produced by fluoride and phosphate. Additions of possible coenzymes were without effect in restoring activity. Slater's studies on stability indicated that the overall system lost activity more rapidly than did succinic dehydrogenase (59).

None of the studies reported in this section have been carried out in terms of oxidative phosphorylation, and the inclusion of the phosphorylative phenomena will no doubt require a reassessment of some of the above data.

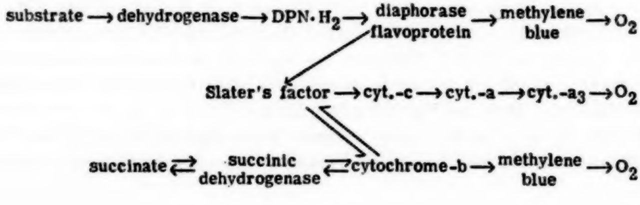
Miscellaneous dehydrogenases.—The dismutation of acetaldehyde to alcohol and acetic acid was formerly ascribed to a "mutase" but Racker (66) was able to show that two separate enzymes, alcohol dehydrogenase and aldehyde dehydrogenase, were responsible. It now appears that "mutases" may be nonexistent.

Glucose dehydrogenase has been further purified by Brunelli & Wainio (67), using lamb liver as a source material. This enzyme requires a carrier system, in contrast to the glucose oxidase(=notatin =penicillin B) obtained from culture media of *Penicillium notatum*, which reacts directly with oxygen forming hydrogen peroxide (68).

Glycerophosphate dehydrogenase activity was found in crystals of myogen A obtained from rabbit muscle, and the two proteins were separated to give crystals of enzyme with constant activity (69). The enzyme catalyses the reaction between reduced DPN and dihydroxyacetone phosphate to give DPN and L-α-glycerophosphate.

Electron Transport Mechanisms

A major advance in this field has come from Keilin's laboratory as a result of studies by Slater (57 to 61). Studies with succinic dehydrogenase (see above) were extended to include the coupling between reduced cozymase and cytochrome-*c* or fumarate. On the basis of studies with BAL it was proposed that a new factor was common to all three systems. The present status of the electron transport system was formulated somewhat as follows, the arrows indicating the electron transfers studied:



It remains to be seen whether Slater's factor is related to the newly discovered cytochrome-*e* of Keilin & Hartree (63). In the above scheme succinic dehydrogenase has been distinguished from cytochrome-*b* although this is not established. Before accepting the scheme, it must be noted that no "diaphorase" was added and that its participation was assumed by Slater. A fact that must be reconciled with the above scheme is the preparation by Heppel (70) and Horecker (71) of the DPN and TPN cytochrome-*c* reductases from animal tissues, since these preparations seem to combine the properties of Slater's diaphorase-plus-factor sequence. Lockhart & Potter suggested in 1941 (72) that the soluble diaphorase might represent a transformation artifact derived from DPN-cytochrome-*c* reductase, and it seems desirable to test the newly isolated reductase for the properties of the Slater factor. Further studies in this field should greatly benefit from Hogeboom's finding (73) that the native DPN-cytochrome reductase appears to be concentrated in the submicroscopic particles (microsomes) of rat liver cytoplasm. While the mitochondria appear to contain both reductase and oxidase activity, the microsomes appear to be relatively low in the oxidase. Mueller & Miller (74) report the occurrence of what appears to be the analogous TPN enzyme in the submicroscopic fraction of rat liver. Horecker & Heppel (75) noted the reduction of cytochrome-*c* by xanthine oxidase in the presence of xanthine, hypoxanthine or acetaldehyde at a rate equal to one half the rate of the direct reaction with oxygen.

Few studies on the coupling of phosphorylation with electron transport *per se* are available but the studies by Lehninger (24, 26, 27), Hunter (21, 22, 23), Green *et al.* (16, 18, 19, 10) and Hummel & Lindberg (29) (cf. p. 3) suggest approaches to the problem, and it is generally recognized that a considerable proportion of the phosphate uptake is associated with the electron transport mechanisms. A thiamine triphosphate has been reported (76).

The breakdown of cozymase has been a great disadvantage in many homogenate systems and the inhibition of the process in brain by nicotinamide has proved very useful. McIlwain & Rodnight (77) point out that the rate of breakdown can actually exceed the rate of respiration on a molar basis. McIlwain (78) has pointed out that $DPN \cdot H_2$ is relatively resistant to cleavage in comparison with DPN, and considers the possibility of regulatory function. New studies showing the importance of the calcium-activated DPN breakdown in homogenates used for succinoxidase assays have been reported by Pardee & Potter (79) who point out that the calcium is probably concerned with the splitting of DPN holo-dehydrogenases, rather than with DPN hydrolysis. Further studies on the calcium effect have been carried out by Leuthardt & Mauron (80).

Cytochrome-*c* and methemoglobin have been found to catalyse the oxidation of adrenaline and various amino and hydroxy-acridines and quinolines to yield compounds which act as hydrogen carriers to promote the oxidation of ascorbic acid. The *o*-quinones and *o*-quinonimines promote the oxidation of cysteine but adrenochrome does not (81, 82).

Oxidases

Tyrosinase and polyphenol oxidases.—Lerner *et al.* (83) showed that mammalian tyrosinase from Harding-Passey mouse melanomas was associated with cytoplasmic particles. They recommend that the separate terms *tyrosinase* and *dopa oxidase* be abandoned in favor of the single term *tyrosinase*, since they felt that both substrates were acted upon by the enzyme. As in earlier work with plant tyrosinase, the induction period in tyrosine depended upon the amount of "dopa" present. Further studies on the granules from mouse melanomas were reported by DuBuy, *et al.* (84). A number of studies on tyrosinase from mushrooms (85 to 88), belladona plants (89, 90), and potatoes (91, 92) have appeared. The suggestion was made that tyrosinase combines two enzyme activities in the same molecule (88) and this appears to be in agreement with other studies.

Studies on the terminal oxidase systems of potatoes (91 to 96) and of green leaves of plants have been reported (97). The respiration of intact potatoes was believed to be carried out by cytochrome oxidase rather than by the phenolase, which required much higher oxygen tensions (95). The oxidase in leaves was studied using glycolic acid and lactic acids. No evidence for either polyphenolase or cytochrome oxidase participation was obtained (97). Sizer (98) reported that poison ivy allergens were inactivated by laccase as well as by tyrosinase.

Crystalline catalase has been obtained from bacteria (99). The same authors prepared crystalline catalase from human erythrocytes, and it was found to have properties almost identical with those of catalase preparations from other animal species (100, 101) contrary to an earlier report. Catalase was shown to be involved in the oxidation of nitrite to nitrate in combination with xanthine oxidase or D-amino oxidase, either in purified recombined systems or in homogenates of rat liver or kidney (102). In the homogenates, the rate was limited by the catalase concentration, which acted as a peroxidase.

Chance has continued his fundamental studies on enzyme-substrate complexes of peroxidase (103, 104) and catalase (105 to 108) including further studies on reaction mechanisms using cyanide to form an enzyme-inhibitor complex (109–110). The most recent paper in the series (108) describes the tentative mechanisms that are proposed for the catalatic and peroxidatic functions of catalase, and is an excellent guide to an understanding of the earlier papers, which are too extensive to be adequately reviewed here.

The two functions of catalase are essentially similar and differ only with respect to the hydrogen donor. A general equation for catalase action involves the following two reactions:

(*a*) $FeOH + H_2O_2$(first molecule = hydrogen acceptor)$\rightleftarrows FeOOH + H_2O$

(*b*) $FeOOH + H_2O_2$(second molecule = hydrogen donor)$\rightleftarrows FeOH + H_2O + O_2$.

Chance (108) points out that these equations are supported by the demon-

stration of the intermediate compound of catalase and hydrogen peroxide, the lack of a Michaelis constant for catalase action, and the peroxidation reactions of the catalase hydrogen peroxide complex. In the peroxidatic reaction the second molecule of hydrogen peroxide can be replaced by any of a number of hydrogen donors including methanol, ethanol, formate, methylene glycol but not acetaldehyde or acetate. It may be helpful to point out that the first and second molecules of hydrogen peroxide in the above equations are called substrate and acceptor respectively by Chance (108) and acceptor and donor respectively by LuValle & Goddard (111). The reviewer prefers the latter terminology which is more in line with current views regarding biological oxidations [cf. (112)], and if it is specified that the compounds are hydrogen-acceptors or donors, no ambiguity should result.

Chance noted that although the above equations had been proposed earlier, it had not been realized that the then accepted Michaelis constant could not be obtained from these consecutive reactions.

It appears that the peroxidative reaction of catalase takes priority over the catalatic reaction under circumstances that might be expected to occur *in vivo* (113). Thus, the physiological function of catalase may be concerned primarily with the reduction of peroxide to water rather than with its oxidation to molecular oxygen.

George (114, 115) reported that concentrations of hydrogen peroxide above about 0.07 M caused a sharp decrease in the rate of the catalatic reaction that could not be attributed to enzyme destruction.

Chance (108) referred to a hypothetical transfer of electrons through the catalase protein as an "internal" reaction, and some theoretical aspects of such a reaction have been taken up by Evans & Gergely (116). Zimmerman (117) discussed "respiratory rebound" in terms of the theory of absolute reaction rates, while Eadie *et al.* (118) discussed kinetic aspects of benzoic acid oxidation.

Inhibitors[3]

Effect of enzyme concentration.—In the classical treatments of competitive inhibition, it has generally been assumed that the amount of the inhibitor i that is combined with the enzyme E is negligble in comparison with the total amount of inhibitor present, so that in the mass law equation, $[(E)(i)]/Ei = K_i$, the value for i is assumed to be equal to the total amount of inhibitor added. However, the fact that certain inhibitors are relatively undissociated from the enzymes with which they combine makes it necessary to consider the effect of enzyme concentration in any studies involving quantitative measurements of the extent of enzyme inhibition (61, 119, 120). When the dissociation constant is sufficiently small, the inhibition may

[3] A large number of papers (over 200) most of which would fall into either the section on Respiration and Assays or the section on Inhibitors have not been cited in this review.

appear to be irreversible, and the inhibitor may actually have the effect of titrating a stoichiometric fraction of the enzyme, so that an inhibitor administered *in vivo* may be able to remain combined with the enzyme molecules through the manipulations necessary for an *in vitro* test of enzyme activity (120). This fact may be of considerable importance in interpreting the *in vivo* effects of such compounds (120). In order to distinguish such inhibitors from truly irreversible enzyme destruction, Ackermann & Potter (120) proposed that they be called pseudo-irreversible inhibitors and suggested a simple means for graphically distinguishing them from the reversible inhibitors by plotting enzyme activity against enzyme concentration; with reversible inhibitors the per cent inhibition was constant at any enzyme concentration, while with the pseudo-irreversible inhibitors, the per cent inhibition depended upon enzyme concentration. Attempts to study these phenomena are complicated by the fact that the inhibitor may combine slowly with the enzyme while the substrate combines rapidly (61, 119, 120). In this situation the relationship between the inhibitor and substrate may involve "interference" if the combinations between enzyme and substrate or inhibitor are mutually exclusive (120).

It appears that most of the sulfhydryl inhibitors used in enzyme studies may be of the pseudo-irreversible type [cf. (121)] especially since the reversal of the inhibition can frequently be accomplished by means of agents that have a higher affinity for the inhibitor than does the enzyme (121). Such inhibitors can exhibit interference phenomena relative to the substrate without necessarily being competitive in type (61, 120).

The inhibition of xanthine (or pterine) oxidase by certain pteridines (119, 122) appears to be an example of this high affinity phenomenon.

Krebs cycle oxidations and phosphorylations.—Several studies of enzyme inhibitors in relation to the Krebs oxidative cycle have appeared. Pardee & Potter noted that oxaloacetate produced a strong inhibition of succinic dehydrogenase only in systems from which ATP was absent (79), and from a study on malonate inhibition (13) they concluded that, at a concentration of 0.004 *M*, succinic dehydrogenase was almost completely inhibited while at higher concentration, the oxidation of oxaloacetate via the Krebs condensation became significantly inhibited in a homogenate system. The inhibitory effect of malonate in slices of rat heart was thoroughly studied by Webb *et al.* (123) who found that malonate stimulated the oxygen uptake at certain critical concentrations and time intervals. It appears possible that their effect was a reflection of an altered phosphorus:oxygen ratio although this aspect was not studied. They also reported inhibitions by fluoride, iodoacetate, cyanide, phlorhizin, and pyrophosphate.

The inhibition of citrate oxidation by a product of the metabolism of fluoroacetate has been mentioned earlier, and appears to constitute a means for a specific block in the cycle. Another reagent for this purpose appears to be *trans*-aconitate, which blocks the action of aconitase (2). Streptomycin has been reported to prevent the Krebs condensation or a closely related reaction (124).

The inhibition of the coupling between phosphorylation and the oxidations of the Krebs cycle has been observed in the case of 2,4-dinitrophenol (DNP), gramicidin, and various other compounds. The relative effects of some 37 compounds related to DNP were studied by Cross *et al.* (16) who found that slight changes in the molecule resulted in the loss of inhibitory capacity. Tepley (20) examined earlier claims by Loomis & Lipmann that DNP substituted for inorganic phosphate in the coupled oxidative phosphorylation and provided substantial evidence that such was not the case. Loomis & Lipmann (125) were able to show that azide also uncouples phosphorylation from oxidation in a homogenate system in which the azide-sensitive oxidative catalysts were replaced by ferricyanide. They referred to similar experiments showing uncoupling by means of methylene blue and cresyl blue. In contrast to compounds that act like dinitrophenol, pentobarbital was shown to inhibit phosphorylation and oxidation of pyruvate in brain homogenates to a similar extent, so that the phosphorus:oxygen ratio remained constant (126).

Sulfhydryl inhibitors—An extensive report on the effect of numerous organic arsenicals on a variety of enzymes showed that pentavalent arsenicals were in general nontoxic until reduced to the trivalent arsenoxides (127), which were considered to form reversible equilibria with thiol enzymes. These inhibitors would be classed as pseudo-irreversible by the reviewer (see above). They were not studied at different enzyme concentrations.

The succinoxidase system was shown to be more sensitive than succinic dehydrogenase to organic mercurials indicating the presence of thiol groups in more than one component of the overall system (128) (Cf. section on DEHYDROGENASES).

A large number of naphthoquinone derivatives (antimalarials)was tested on a succinoxidase preparation but no correlation with therapeutic effects resulted (129). Although tests at different enzyme concentrations were not reported, portions of an inactive preparation "caused a progressive increase in the drug required to half inhibit the system," suggesting that the per cent inhibition would have varied with changes in enzyme concentration. In this study a comparison with a standard drug was always made. A better correlation with therapeutic action was obtained when the drugs were tested as inhibitors of the respiration of parasitized red blood cells (130).

Miscellaneous.—The previously reported toxicity of ferrous iron for the glycolytic enzymes in mouse brain homogenates was traced to a cathepsin-like factor present in normal brain. The factor was shown to be activated by ferrous iron, and prevented from acting by L-leucinamide and some related compounds (131). Activation of the factor, resulting in the destruction of glyceraldehyde phosphate dehydrogenase, was given as the probable explanation of the inhibition of glycolysis by certain virus preparations. There was no evidence that the factor was operating in brain homogenates under the conditions ordinarily employed for the study of glycolysis, nor was any data given to indicate the possible effects of the "factor" preparation under these conditions.

The effect of synthetic estrogens upon the succinoxidase system has been reinvestigated by Case & Dickens (132) who found no correlation between hormonal and enzyme-inhibitory action. The compounds did not uniformly attack a single component of the succinoxidase system and 4,4-dihydroxystilbene in particular, appeared to act upon an unknown component (Slater factor?). Shacter (133) studied the effects of diethylstilbestrol and several natural hormones upon yeast respiration, which was accelerated in the absence of glucose but inhibited in the presence of glucose.

An unusual inhibition by low concentrations of inorganic phosphate or arsenate has been reported for L-amino oxidase of snake venoms (134).

Respiration and Assays[3]

The oxidation of endogenous unsaturated fatty acids was studied in normal and scorbutic guinea pig tissues by means of the thiobarbituric acid color reaction, which is based upon the combination of the reagent with a 3-carbon fragment that is presumed to arise from linolenic acid or a closely related compound. Tissues from deficient animals gave much lower color values than those from normal animals and were restored to normal in most cases by the addition of ascorbic acid *in vitro* (135).

Ascorbic acid deficiency leads to the excretion of partially oxidized tyrosine metabolites and it was shown that tyrosine was oxidized by liver slices from normal guinea pigs but not from scorbutic animals. Sealock & Goodland (136) next turned to homogenates and reported the uptake of four atoms of oxygen per mole of tyrosine by homogenates of normal liver. They also reported activation of dialysed extracts by boiled extracts. Clegg & Sealock (137) studied the oxidation of dihydroxyphenylalanine in extracts from guinea pig kidney homogenates. In marked contrast to results with kidney slices, there was no difference in the oxidative activity of kidney extracts from normal and scorbutic animals on this substrate. No explanation of this difference was available. Analysis of reaction products showed that hydroxytyramine (a hypertensive substance) was formed and the catechol nucleus was then converted to an unknown product. The α and β carbons of tyrosine and two of the ring carbons were shown to be converted to acetoacetate in rat liver slices by isotope techniques (138, 139). In rats, a pteroylglutamic acid (PGA) deficiency induced by feeding either succinylsulfathiazole or 4-amino-PGA led to decreased rate of tyrosine oxidation by liver homogenates, and some restoration was achieved by adding back PGA *in vitro* or *in vivo*. In contrast to these results, Keith *et al.* (140) were unable to show a change in the tyrosinase content of livers from PGA deficient chicks. However, the xanthine oxidase was increased nearly three-fold in the deficient chicks, while chicks on commercial diets showed values lower than found with the supplemented diets. In rats, (141) addition of PGA to a purified diet also lowered the xanthine oxidase of the livers, but animals on a stock diet showed higher values (142). Since the PGA used in the chick experiments contained an impurity capable of inhibiting xanthine oxidase (140) the above observations may be related to studies by Lowry *et al.* (119,

143) since these workers obtained an inhibitory breakdown product from PGA with such small dissociation constants that it may be capable of combining with the enzyme *in vivo* and affecting the activity of the enzyme in the *in vitro* test. (See section on INHIBITORS.) Lowry *et al.* showed that xanthine oxidase, which appears to be identical with pterine oxidase, will oxidize xanthine, xanthopterine and 2-amino-4-hydroxypteridine. It is inhibited appreciably by as little as 10^{-4} μg per ml. of 2-amino-4-hydroxy-6-formylpteridine, which has an inhibitor constant of approximately 0.6×10^{-9}. They pointed out that pteroylglutamic acid is known to reduce or eliminate the adenine requirement of certain microorganisms and pointed out that if the 6-formyl compound were released slowly from (PGA) in tissues it might tend to preserve hypoxanthine or adenine from oxidation. They showed that when PGA is irradiated with ultraviolet light in slightly acid solution, it undergoes oxidative cleavage to yield in succession 2-amino-4-hydroxy-6-formylpteridine, 2-amino-4-hydroxy-6-carboxypteridine, and 2-amino-4-hydroxy-pteridine (143), which were used in the above studies.

METHODS

New studies on the factors affecting the maintenance of respiration have been carried out using slices of heart (144, 145) and kidney (146) and homogenates of kidney (14). Only in the kidney slices was it possible to maintain the original rate for 4 hr. (146). This important result was based on a new medium which contains phosphate, glucose, and calcium in physiological amounts, and has no added bicarbonate. The solution is almost identical with Krebs-Ringer-phosphate except that it contains about one-fourth as much phosphate. When either or both the calcium and glucose were not present, the Q_{O_2} was not maintained. The medium does not lose calcium by precipitation as in the case of most similar media and has less buffering capacity than media generally used. The success with kidney is undoubtedly related to the capacity of kidney tissue to oxidize completely the acids produced during glycolysis and will not necessarily be duplicated in other tissues.

The use of homogenates has become very widespread and there has been considerable diversity in the media used for homogenization and subsequent studies. The effect of adding ATP to reaction mixtures involving homogenates has had such remarkable effects that it has become appropriate to test its effects and stability in almost any system involving homogenates. Several studies in this laboratory (14, 147) indicate that, as might be expected, the enzymes in a homogenate are subject to action by destructive enzymes that are presumably segregated in intact cells and no work with homogenates can afford to overlook this fact, which does not however preclude the use of these preparations for many enzyme studies.

The question of how to prepare homogenates is an important one, and the use of the Waring blendor has been questioned, although it was not compared with the Potter-Elvehjem type (148, cf. 12). New types of blendors have been proposed (149, 150).

Ellinger & Quastel have studied the respiration of microorganisms sus-

pended in thin films of liquid on solid particles (151). Pardee (152) has described a new method for absorbing carbon dioxide from Warburg flasks so as to permit measurements of oxygen uptake at various carbon dioxide tensions, using ethanolamine: hydrochloric acid mixtures instead of potassium hydroxide in the center well.

New methods have appeared for the preparation of aconitase (153,) uricase (154), phosphocreatine (155), diphosphopyridine nucleotide (156, 157), and triphosphopyridine nucleotide (158). The effect of trace impurities in ATP has been noted (159).

New or modified methods have been described for cytochrome oxidase (160, 161, 162), peroxidase (163, 164, 165) phenol oxidase (166) catalase (167) oxaloacetic acid oxidase (10), succinic dehydrogenase (168) and diphosphopyridine nucleotide (169, 170).

Analytical methods of interest in connection with biological oxidations include partition chromatography for fumaric acid and related compounds (171), a fluorimetric determination of malic acid (172), a colorimetric method for acetate and fluoroacetate (173), a study on the specificity of the pentabromoacetone method for citric acid (174), a manometric method for glucose using glucose oxidase (175), improvements in the manometric estimation of succinic and α-ketoglutaric acids (176) a spectrophotometric method for determining protein (177), and anion-exchange chromatography methods for mononucleotides (178).

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PROTEOLYTIC ENZYMES[1]

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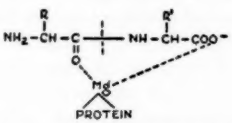
Exopeptidases

During the past few years it has become increasingly obvious (1, 2) that a large number of the exopeptidases are activated by metals. This year Smith (3) formulated a theory of the mode of action of metal-containing exopeptidases. His theory assumes that the enzyme-substrate complex is a chelation complex formed through the metal. According to Smith, the case of carboxypeptidase may be represented as follows:



The chelation linkages which enclose the sensitive bond produce an electronic distortion which results in the labilization and hydrolysis of the peptide bond. The theory is in agreement with the known facts of optical specificity. The extension of this theory has been presented as part of a symposium on amino acid metabolism (4).

In the original Bergmann & Fruton classification (5, 6) aminopeptidases were defined as enzymes hydrolyzing the peptide bond adjacent to a free amino group, and carboxypeptidases as hydrolyzing the peptide bond adjacent to a free carboxyl group, regardless of the length of the chain. Only one representative of each group was known at that time. In 1942 Bergmann (6) wrote:

> One must expect to find in the future not only one but several aminopeptidases which should differ in their side group specificity and, similarly, to encounter several carboxypeptidases of distinct side group specificity.

This prediction has been fulfilled. As to the number of individual enzymes in each group, the reviewer prefers not to commit himself on the basis of the available evidence, and to withhold judgment until the respective enzymes are isolated. For the time being, it seems most convenient to specify the enzyme by the substrate on which activity was measured and to go no further.

Side group specificity has been extensively investigated only in the case of the crystalline carboxypeptidase of Anson (7). Our knowledge of other members of the peptidase family in this respect is still very incomplete. It may be interesting to note, however, that at least in two cases the minimal

[1] This review deals mainly with the period from November 1, 1948 to November 1, 1949.

length of the peptide chain constitutes one of the specific requirements. On the other hand the existence of a nonspecific dipeptidase, already questioned by Bergmann (6), is becoming increasingly doubtful (2). It appears, therefore, that the length of the chain may be one of several requirements for specificity, not however unique.

Carboxypeptidase of Anson.—Smith & Hanson (8) investigated the action of several metal poisons on crystalline carboxypeptidase prepared according to the method of Anson (7). The results indicate that carboxypeptidase is a metallo-protein, the metal being firmly bound, unlike that in other peptidases. Magnesium has been tentatively identified as the metal of carboxypeptidase, since it was found by spectrophotometric analysis in the ash of the enzyme preparation.

Elkins-Kaufman & Neurath (9) determined the structural requirements for the specific inhibitors of carboxypeptidase, using carbobenzoxyglycyl-L-phenylalanine as substrate and D-phenylalanine as inhibitor. The structural elements of the latter compound which may be responsible for inhibition were analyzed. The α-amino group, the carboxyl group, and the phenyl ring were foci of enzyme-inhibitor interaction. Carboxypeptidase (10) hydrolyzed peptides containing a terminal L-methionine at a somewhat slower rate than peptides containing a terminal phenylalanine or tyrosine group.

Hanson & Smith (11) have found that carboxypeptidase has no hydrolytic action on either carbobenzoxyglycyl-D-tryptophane or carbobenzoxy-D-tryptophylglycine. This finding signifies that in the molecule of substrate the optical configuration of the amino acid on either side of the susceptible linkage must be L.

Smith *et al.* (12) determined the sedimentation constant of crystalline pancreatic carboxypeptidase to be 3.07 Svedberg units, and calculated the molecular weight as 33,800. The inactivation of carboxypeptidase by α-radiation and x-rays has been studied (13). The x-ray ionic yield was approximately constant over a wide range of enzyme concentration and was equal to about 0.18 enzyme molecule (molecular weight, 35,000) inactivated per ion pair of water. The α-ray yield was only 1/20 of the x-ray ionic yield.

A manometric method of determination of carboxypeptidase using chloroacetyltyrosine or carbobenzoxyglycyl-L-phenylalanine as substrates has been described by Zittle (14).

The utilization of histozyme (an enzyme possibly of the carboxypeptidase type) for the resolution of racemic amino acids has been known for a long time (15). This work was revived last year in Greenstein's laboratory (16). Fodor *et al.* (17) purified an enzyme from kidney and used it as a tool for the preparation of optically active alanines. A racemic mixture of N-acetyl-DL-alanine was subjected to the action of the enzyme and the resulting free L-alanine was precipitated with acid alcohol. The N-acetyl-D-alanine remaining in solution was later hydrolyzed by acid, and D-alanine was recovered.

Using chloroacetyl derivatives, Price *et al.* (18) succeeded in applying the same principle to the resolution of racemic mixtures of the following amino acids: methionine, valine, threonine, isoleucine, serine, leucine, aspartic acid,

and glutamic acid. Gilbert *et al.* (19), using a purified pancreatic carboxypeptidase and chloroacetyl derivatives, resolved racemic mixtures of phenylalanine, tyrosine, and tryptophane. They noted that acetyl derivatives of aromatic amino acids were very resistant to the action of carboxypeptidase. The application of papain to the resolution of DL-methionine was reported a year earlier by Fruton and his co-workers (see p. 31).

Conjugases.—This is a general and rather unfortunate term for the enzymes splitting conjugated forms of pteroylglutamic acid. The early work (20, 21) indicated that there were at least two types of conjugases: the first having a pH optimum of about 4.5, represented by hog kidney conjugase (22), and the second having a pH optimum of about 7 and represented by chicken pancreas conjugase (23). The elucidation of the chemical nature of conjugates and the fact that the methyl ester of pteroylheptaglutamate was not susceptible to hydrolysis led to the classification of conjugases as members of the group of carboxypeptidases (24). It soon became obvious that neither of the conjugases is identical with the carboxypeptidase of Anson (7), since the latter has no conjugase activity.

Studies on the specificity of the partially purified chicken pancreas conjugase (25) originally indicated that only the γ-peptides of pteroylglutamic acid were susceptible to the hydrolytic action of the enzyme. More recently (26) it was found that pteroyl-α,α-triglutamate was also attacked by chicken pancreas conjugase, but at a considerably slower rate than pteroyl-γ,γ-triglutamate. The final formulation of other requirements for the molecule of substrate for chicken pancreas conjugase would be premature, since only a few synthetic peptides have been investigated. From the data now at hand, it would appear, however, that a minimum of three terminal glutamic acid residues is required, since neither pteroyl-α-diglutamate nor pteroyl-γ-diglutamate was attacked (26). Consequently, the diglutamate and not the monoglutamate would represent the end-product of the enzymatic digestion.

The structural requirements for the substrate for hog kidney conjugase are even less known. Mims & Bird (27) have accumulated evidence that hog kidney conjugase is composed of two different enzymes. The hydrolysis of pteroylheptaglutamate required the presence of both components, while the hydrolysis of pteroyltriglutamate could be accomplished by one. No evidence for two components in chicken pancreas conjugase was found (26). Chicken pancreas conjugase requires calcium for activation while no metal is required by hog kidney conjugase. The conclusion that chicken pancreas conjugase and hog kidney conjugase have different substrate specificities was supported by Sreenivasan, Harper & Elvehjem (28), who found that stepwise hydrolysis of the substrate by the two enzymes gave much higher values for folic acid than when the enzymes were used singly. A similar conclusion was reached by Couch, Panzer & Pearson (29) who used chicken pancreas conjugase and chicken egg conjugase.

A conjugase with an optimum pH close to 7 has been found in the blood of several animal species, including man (30). A conjugase active below pH

7 has been reported in human plasma (31, 32, 33). No change in conjugase was observed in livers of chicks receiving graded amounts of pteroylglutamic acid in the diet (34). An enzyme, which at pH 2.0 does not liberate free pteroylglutamic acid from pteroylheptaglutamic acid but changes it into an unknown product, has been detected in normal gastric juice (35, 36).

Aminopeptidases.—Patterson *et al.* (37) investigated the peptidases of the salivary glands of *Drosophila.* Glycerol extracts of the gland hydrolyzed the following peptides: alanylglycine, leucylglycine, leucylglycylglycine, glycylglycine, and diglycylglycine in descending order of velocity. Of several metals tried only manganese produced activation and only with leucylglycylglycine as substrate. The authors believe that at least four different enzymes are responsible for these reactions: alanylglycine dipeptidase, leucylglycine dipeptidase, leucine aminopeptidase, and aminopolypeptidase.

The same authors have found (38) that the amount of alanylglycine peptidase increased parallel to the increase in total nitrogen during the stage of prepupal instar, but decreased during the period of histolysis of the gland. During the period of final larval growth, the content of peptidase also paralleled the total nitrogen. This was interpreted as evidence that alanylglycine peptidase is associated with protein synthesis and the growth of the cell. Speculations concerning the relationship between pentose nucleic acid and peptidase activity were included.

Peptidases attacking leucylglycine, leucylglycylglycine, and chloroacetyl tyrosine were found by Lichtenstein (39) in glycerol extracts of eggs and larvae of *Bombyx mori.*

An extensive study of the specificity of leucine aminopeptidase has been made by Smith & Polglase (40). It was shown that in addition to L-leucinamide several other aliphatic L-amino acid amides were hydrolyzed. The enzyme shows a marked specificity toward the residue possessing the free amino group, since D-leucinamide, D-leucylglycine, D-alanyl-L-leucinamide, and β-alanyl-L-leucinamide were not attacked. L-leucyl-L-alanine was hydrolyzed 23 times faster than L-leucyl-D-alanine. The leucine aminopeptidase of serum (41) was found to increase after fractures of the bone.

Contrary to previous observations (42), Schwartz & Engel (43) have found that serum peptidase (substrate: leucylglycylglycine) was not increased after the injection of either cortical extracts or adrenocorticotropic hormone. The peptidase activity, however, was correlated with the degree of hemolysis. Johansen & Thygesen (44) applied a dilatometric technique in their study of blood peptidases. The technique offered the advantage of continuous observation and was equally as accurate as the titrimetric method. The following racemic peptides were used as substrates: glycylglycine, alanylglycine, leucylglycine; diglycylglycine, alanylglycylglycine, leucylglycylglycine; leucyldiglycylglycine. Erythrocytes contained 40 to 50 times more enzyme than serum and probably constituted the essential source for serum peptidases.

The action of crude animal dipeptidase on DL-leucylglycine and glycyl-DL-leucine has been studied (45). Only the L-form was hydrolyzed, the D-peptide

remaining unchanged. The enzyme was activated by manganese and cobalt.

An enzyme hydrolyzing L-leucylglycine and probably different from leucine aminopeptidase has been described by Smith (2, 46). Croxatto *et al.* (47) have reported leucylglycinase in rabbit blood, and found that it was increased during tourniquet shock.

As an extension of their previous work Roulet & Zeller (48) applied venom amino acid oxidase to the determination of L-peptidases in *Mycobacterium tuberculosis* and related strains. Both the intact bacteria and their homogenates were capable of splitting the following peptides: glycyl-L-leucine, L-leucyl-glycine, L-leucylglycylglycine, glycyl-L-tyrosine, leucyl-L-tyrosine, and glycyl-L-tryptophane. The authors believe that the first three peptides listed are split by three different enzymes.

A new enzyme, carnosinase of swine kidney, has been partially purified by Hanson & Smith (49). The enzyme required activation by a metal, either zinc or manganese, and hydrolyzed carnosine (β-alanyl-L-histidine), D- and L-alanylhistidine, glycyl-L-histidine, and glycyl-L-histidinamide. D-carnosine, L-aspartyl-L-histidine, carbobenzoxy-L-carnosine, and carbobenzoxyglycyl-L-histidinamide were relatively resistant to the action of the enzyme.

Lederer & Lourau (50) described the action of several cations on an enzyme, possibly a peptidase, secreted by the gastric mucosa and having the characteristics of the intrinsic factor of Castle. They found that low concentrations of lead inhibited the enzyme, and that the inhibition was reversed by either nickel or cobalt.

Dehydropeptidases.—The differentiation of dehydropeptidases into dehydropeptidase I (specific substrate: glycyldehydroalanine) and dehydropeptidase II (specific substrate: chloroacetyldehydroalanine) has already been reviewed (2, 51). The original data, however, have only recently been published (52). Dehydropeptidase I was found to be associated with the particulate matter of the cell and was purified about 25-fold, using partial digestion with trypsin. Dehydropeptidase II was purified about 1,000-fold and was found to be homogeneous in the ultracentrifuge.

Fodor *et al.* (53) studied the enzymatic hydrolysis of saturated and unsaturated peptides by crude kidney extract. Chloroacetylglycyl-L-alanine and chloroacetylglycyldehydroalanine were completely hydrolyzed by kidney extract. The acyl-peptide bond was resistant in compounds such as chloroacetylsarcosyl-L-alanine and chloroacetyl-D-phenylalanylglycine. The terminal peptide bond was resistant in compounds like chloroacetylglycyl-D-alanine and acetylglycyldehydrophenylalanine. It is not yet clear whether the kidney enzyme splitting tripeptides is identical with any of the previously described dehydropeptidases acting on dipeptides.

Greenstein & Leuthardt (54) found dehydropeptidase activity in aqueous extracts of various mouse and rat tumors. The rate of hydrolysis for alanyldehydroalanine was very high, and of the same order of magnitude as that of kidney, the most active normal tissue. The rate of hydrolysis of glycyldehydroalanine was somewhat lower, and was almost negligible for glycyldehy-

drophenylalanine and N-methylglycyldehydroalanine. The addition products of dehydropeptides have been described (55).

Endopeptidases

Chymotrypsins.—A new method for preparing salt-free crystalline chymotrypsinogen-α and chymotrypsin-α from solution in dilute ethyl alcohol has been described by Kunitz (56). Both proteins were first crystallized according to the method of Kunitz & Northrop (57), then dialyzed free of salt, and recrystallized from alcohol. In agreement with Jacobsen (58) and Brown *et al.* (59), Kunitz (56) observed that chymotrypsinogen-α can be crystallized from aqueous solutions at pH 5.

MacAllister, Harmon & Niemann (60) have found that a 0.00322 *M* solution of N-benzoyl-DL-tyrosylglycinamide was hydrolyzed by chymotrypsin-α at about the same rate as a 0.00177 *M* solution of the corresponding L-peptide, Since the DL-peptide is much more readily available, this finding is of practical importance. The nature of the inhibition by the D-isomers was investigated on the more readily soluble substrate, N-acetyl-L-tyrosylglycinamide. The D-isomer produced little inhibition at 0.0017 *M* concentration but considerably more at 0.1 *M*, indicating that the inhibition is of a competitive nature, and a function of the relative concentrations of the DL-mixture and enzyme.

Kaufman, Neurath & Schwert (61) made an extensive study of the kinetics of the peptidase and esterase activities of chymotrypsin-α. The most specific substrates found were: benzoyl-L-tyrosinamide for the peptidase activity and benzoyl-L-tyrosine ethyl ester for the esterase activity. The latter substrate was hydrolyzed at a considerably higher rate.

Snoke & Neurath (62) and Kaufman & Neurath (63) defined the main structural requirements for substrate specificity in relation to chymotrypsin-α as follows: (*a*) a secondary peptide group, or a group capable of hydrogen bond formation by hydrogen donation; (*b*) an enzymatically susceptible peptide, amide, or ester bond, and (*c*) a tyrosyl, phenylalanyl, tryptophyl, or methionyl residue attached to the α-carbon atom. The inhibition of chymotrypsin-α by structural analogues either devoid of the susceptible bond, or containing a bond resistant to enzymatic hydrolysis, has been reported (64).

The effect of methanol on the hydrolysis of acetyl-L-tyrosinamide was studied (65). In several previous instances Neurath and co-workers have postulated that the apparent rate of the enzymatic reaction results from two processes: the formation of a stable enzyme-substrate complex, followed by the activation of this complex. Analyzed in terms of the two process-component system, it was found that methanol lowered the rate of combination of the free enzyme and substrate, but had no effect on the rate of activation of the stable enzyme-substrate complex.

Schwert (66) has found the sedimentation constant for chymotrypsinogen-α to be 2.7 Svedberg units. Both the α- and the γ-chymotrypsins associ-

ate to form dimers. The $S_{20,w}$ values for the dimers are 3.5 for the α-form and 3.2 for the γ-form. The molecular weights for chymotrypsinogen, the monomer of α-chymotrypsin, and the monomer of γ-chymotrypsin are 22,000, 17,500, and 16,500, respectively.

Peters & Wakelin (67) have found that thiol compounds act on trypsin, α-chymotrypsin, and α-chymotrypsinogen by liberating SH groups, and that this reaction cannot be reversed by exposure to S-S compounds. α-Chymotrypsinogen was precipitated by monothioethylenglycol, while trypsin and chymotrypsin were inactivated.

A very interesting study of the inhibition of the proteinase and esterase activities of both trypsin and chymotrypsin was reported by Jansen *et al.* (68). Three inhibitors were studied: diisopropyl fluorophosphate, tetraethyl pyrophosphate, and diethyl *p*-nitrophenyl thiophosphate. Only the first had strong inhibitory action. In all but one case, both the protease and the esterase activities were inhibited to the same degree, indicating that the same groups in the molecule are responsible for both activities. No difference was found in the behavior of α- and β-chymotrypsins. A sample of chymotrypsin-α (assumed molecular weight, 27,000), after being completely inhibited by an equimolar amount of diisopropyl fluorophosphate, was crystallized by the procedure used for the active enzyme. The crystals, indistinguishable from the active chymotrypsin, were recrystallized, dialyzed, and lyophilized, the enzyme remaining completely inactive. Further work (69) using diisopropyl fluorophosphate containing P^{32} led to the isolation of the crystalline inactive α-chymotrypsin containing 1.1 moles of phosphorus per mole of enzyme. Chymotrypsinogen under the same conditions did not react with the inhibitor.

"Hence, the conversion to the active enzyme liberates not only the groups responsible for activity but also those with which diisopropyl fluorophosphate reacts, the two possibly being the same."

The discovery of new forms of chymotrypsin by Jacobsen (58) and by Laskowski and co-workers (70, 71, 72, 59) was reviewed last year (2). According to Jacobsen, chymotrypsins π and δ result upon the cleavage of one and two peptide bonds, respectively, in chymotrypsinogen-α. Schwert & Kaufman (73) prepared the δ-form (the more stable of the two) and investigated its specificity and sedimentation behavior. They found, as should be expected from the observations of Jacobsen, that δ-chymotrypsin represents an intermediate form between α-chymotrypsinogen and α-chymotrypsin. They also found the substrate specificity requirements to be identical with those of the α-form. The increased peptidase activity of the δ-form was accompanied by a parallel increase in esterase activity.

In the reviewer's laboratory the previous work on chymotrypsin(ogen)-B (70, 71, 72, 59) has been extended to electrophoresis and solubility studies (74). Electrophoretically, the proteins of the B-series were found to be equally as pure as the proteins of the α-series. The solubility studies, however,

revealed the presence of small amounts of impurities. The proteins of the B-series were distinctly different from the proteins of the α-series with respect to the isoelectric point. In both cases, however, the isoelectric point of the active enzyme was at a lower pH than the isoelectric point of the corresponding zymogen. In confirmation of the results of Anderson & Alberty (75), and contrary to those of Kunitz (76), the isoelectric points for α-chymotrypsin-(ogen) were found to be 8.3 and (9.1), respectively.

From the data available at present on synthetic substrates, the specificities of chymotrypsin-B and chymotrypsin-α are identical (77). Both enzymes act on casein in a similar manner, the rate of hydrolysis being somewhat slower for chymotrypsin-B. The only difference in action of the two forms of chymotrypsin noted thus far (78) has been the inability of chymotrypsin-B to destroy "paramecin."

In addition to the use of papain (10, 79) and carboxypeptidase (16 to 19), chymotrypsin has been used for the resolution of racemic amino acids (80, 81, 82). The racemic DL-methyl ester of tryptophane was digested with crystalline α-chymotrypsin (82). Free L-tryptophane crystallized out, while the untouched D-tryptophane methyl ester was recovered from the solution. The same principle was applied to the resolution of isopropyl esters of DL-methionine (81).

A turbidimetric method for the determination of proteolytic activity has been described (83). The synthetic action of chymotrypsin was reported by Tauber (84). The use of chymotrypsin in the treatment of cancer has been proposed (85, 86) and the mechanism of action has been discussed (87, 88, 89), but the effectiveness of such treatment has been completely denied (90, 91).

Pepsin.—Dieu & Bull (92) determined the film molecular weight of pepsin as 32,400 and found that pepsin molecules do not dissociate on the surface. Bersin & Berger (93) have found that the digestion of edestin by pepsin was inhibited by oxalate, phosphate, succinate, borate, desoxycholate, and metaphosphate; no inhibition was observed with either brucin or cinchonin. The digestion of casein by trypsin was inhibited by putrescin, thiamine, veratrin, strychnin, brucin, and cinchonin, but not by anions. This was interpreted to mean that salt-like linkages are involved in the formation of the enzyme-substrate complex.

Dekker, Taylor & Fruton (10) synthesized several new peptides of methionine. They found that pepsin hydrolyzed carbobenzoxy-L-methionyl-L-tyrosine and L-methionyl-L-tyrosine. The last finding indicates the necessity for the revision of the classical views (5) concerning the specificity of pepsin.

Bull & Currie (94) investigated the rate of digestion of crystalline egg albumin by crystalline pepsin as a function of substrate concentration, of pH, and of temperature. These workers conclude that the activated complex is not identical with the Michaelis-Menton complex. The rate of activation of the latter is critical for the total rate of reaction. The direct participation

of the hydrogen ion in the formation of the activated complex is also postulated.

The turbidimetric determination of peptic activity has been described (95). The addition of sulfhydryl-containing compounds slightly accelerated the rate of hydrolysis of egg albumin by pepsin (96).

In continuation of his previous work (97) which was reviewed last year, Buchs (98) published a paper presenting evidence that in gastric juice cathepsin is equally as abundant as pepsin. Cathepsin is present as zymogen and is activated by hydrochloric acid. The identification of the enzyme as cathepsin is based on the pH optimum and activation by sulfhydryl reagents.

The presence of a second proteinase in gastric juice has been confirmed by Hunt (99) who could not, however, confirm the existence of a pepsin inhibitor.

Trypsin.—On previous occasions Linderstrøm-Lang and co-workers have expressed the view that proteolysis is preceded by denaturation of the substrate. A new attempt to supply experimental evidence in support of this claim has been made (100) with partial success. In a system composed of β-lactoglobulin and trypsin, the denaturation of the substrate increased the rate of proteolysis, and conversely, the addition of trypsin increased the rate of denaturation of β-lactoglobulin by urea solutions. These findings could not be confirmed when either serum albumin or egg albumin was substituted for β-lactoglobulin.

It has been found that N-benzoyl-L-arginine inhibits the tryptic hydrolysis of N-benzoyl-L-argininamide (101, 102). The hydrolysis of methyl, ethyl, isopropyl, benzyl, and cyclohexyl esters was not inhibited by the presence of the free peptide.

The work of Fraenkel-Conrat *et al.* (103) on the interaction of trypsin with the egg white inhibitor was reviewed previously. In continuation of this work Lineweaver *et al.* (104) have described a method which permits the determination of trypsin in the presence of ovomucoid, and vice versa. The method is based on the fact that acetylated trypsin is not inhibited by either ovomucoid or acetylated ovomucoid. By this method it was possible to establish that egg white contains less than 0.004 per cent trypsin, if any.

Volgunov (105) investigated the action of trypsin and the proteinases from pea sprouts on air-dried peptone, which was equilibrated with atmospheres containing 75 per cent and 91 per cent moisture, respectively. The rate of hydrolysis was equal at both levels of moisture. Trypsin has been used for the extraction of plant growth substances from plant tissue (106).

Trypsin inhibitors.—The following naturally-occurring trypsin inhibitors are known: (*a*) the original crystalline pancreatic inhibitor of Kunitz & Northrop (107); (*b*) the crystalline pancreatic inhibitor of Kazal *et al.* (108); (*c*) a partially purified trypsin inhibitor from blood (109); (*d*) the crystalline soybean trypsin inhibitor of Kunitz (110); (*e*) the crystalline lima bean trypsin inhibitor of Tauber *et al.* (111); (*f*) a purified ovomucoid (egg white inhibitor) (112), and (*g*) a partially purified inhibitor from colostrum (113).

This list seems likely to be extended in the near future to include the inhibitors for other proteases. Furthermore, it seems to the reviewer that the work on naturally-occurring inhibitors is likely to become one of the most fertile fields in the biochemistry of tomorrow. Judging from the results obtained with the two-component system (substrate-enzyme), the three-component system (substrate-enzyme-inhibitor) should be equally fruitful.

Although the work of Kunitz (114) on the general properties of soybean trypsin-inhibitor has been reviewed previously, attention is here called to his discovery of two new types of inhibition. The first is represented by a stoichiometric, undissociable compound formed by trypsin inhibitor with trypsin, and the second by a reversibly dissociable complex obtained with chymotrypsin. It is surprising how little this finding has been utilized for the purpose of identification of either enzymes or inhibitors.

In continuation of his work Kunitz (115) studied the kinetics and thermodynamics of reversible denaturation of crystalline soybean trypsin-inhibitor. In the temperature range, 35° to 50°, a solution of protein consists of a mixture of native and denatured forms in equilibrium with each other. The reversibility of denaturation appears to be complete. Thermodynamic data for the energies of reaction and activation were presented.

An interesting application of soybean trypsin-inhibitor to the culture of tissue *in vitro* has been reported (116, 117). Certain types of tissue (including Rous sarcoma) are difficult to grow, since they liquify clots of homologous plasma. The addition of crystalline (116) or purified (117) soybean trypsin inhibitor prevents liquefaction. The addition of the inhibitor to the medium also results in some inhibition of growth, which, however, could be restored to normal after passing the tissue three to five times through media containing inhibitor.

The lima bean inhibitor, recently crystallized by Tauber *et al.* (111), has many properties similar to soybean inhibitor, with the exception that it is heat stable. It also shows some inhibitory action toward chymotrypsin.

Duthie & Lorenz (118) investigated serum trypsin-inhibitor viscosimetrically, with gelatin as substrate. Serum inhibited trypsin by forming a reversibly dissociable complex, time being required for this reaction. Fractionation of the serum was attempted, resulting in a slight concentration of the inhibitor in the albumin fraction. The authors were unsuccessful in purifying the inhibitor by the method of Schmitz (109). The presence of another much more labile inhibitor, which is active against bacterial proteases, but is different from trypsin inhibitor, was described (119). The presence of inhibitors for α-chymotrypsin and for rennin in human plasma has been claimed (120).

Nutritional significance of trypsin inhibitors —Several papers dealing with the nutritional significance of soybean and lima bean trypsin-inhibitors have appeared in the past year. In confirmation of the previously reviewed results of Klose *et al.* (121), Westfall *et al.* (122) reported that feeding of "protolysate" (an incomplete enzymatic digest of casein) together with an extract

of raw soybean (crude trypsin inhibitor) resulted in a decrease in growth, indicating that the deleterious effect of the extract was not necessarily due to its effect on digestion. Partially purified soybean trypsin-inhibitor showed (123) little inhibitory action on growth. The prolonged feeding of raw soybean meal (124) induced in chicks an enlargement of the pancreas and an increase in its proteolytic content.

Liener & Fevold (125) believe that the antitryptic factor does not fully account for the differences in release of amino acids from raw and autoclaved soybean meal by commercial pancreatin. The action of commercial pepsin and trypsin on soybean protein has been studied (126). Boiling caused no change in the release of amino acids from soybean meal by acid hydrolysis, but resulted in a marked increase when crude pancreatic extracts were used as hydrolytic agents (127). Autoclaving of soybean meal caused the binding of variable amounts of methionine in a form from which biologically active methionine was liberated by acid hydrolysis but not by enzymic hydrolysis *in vitro* (128). Optimal growth-promoting values for soybean meal were achieved by heating for 20 to 30 min. at 15 pounds pressure (129).

Klose *et al.* (130) investigated a partially purified growth depressing factor from lima beans which also possessed trypsin inhibiting properties. At least part of the growth inhibiting properties was destroyed by heat. Since then, Tauber *et al.* (111) have crystallized a heat resistant inhibitor from lima beans. Until a careful comparison of the heat lability of crystalline and crude material is made, the conclusion concerning the existence of one or more growth inhibiting factors in lima beans appears to be premature.

Intracellular plant proteinases.—The method of Dekker & Fruton (79) for the preparation of optically active amino acids has been reviewed. This method is based on the incubation of N-acetyl-DL-amino acid with aniline in the presence of cysteine-activated papain. Only the L-anilide is formed and crystallizes out, while N-acetyl-D-amino acid is recovered from the solution. This principle was applied by Reed *et al.* (131) to the preparation of optically active isomers of S-benzoylhomocysteine with excellent results. The same principle was used by Hanson & Smith (11) for the resolution of DL-tryptophane, and by Polglase & Smith (132) for the preparation of carbobenzoxy-D-alanine. Finally, Dekker, Taylor & Fruton (10) have used this method for the preparation of several new peptides of methionine.

The protease content of tobacco leaf (133) was decreased when nitrogen-containing fertilizers were added to the soil in which the plants were grown and increased when phosphorus-containing fertilizers were added. Local infection with tobacco mosaic virus (134) had no effect while a systemic infection produced significant enhancement of the positive effect of phosphorus fertilizer and the negative effect of nitrogen fertilizer on protease content. A method for the removal of proteolytic enzymes from preparations of amylolytic enzymes (135) has been described. Denaturation of albumin by urea was found to increase its susceptibility to the action of papain (136).

Intracellular animal proteinases.—The activity of cathepsin II against

benzoylargininamide was determined in minced livers of fed and fasted rabbits (137). Since the ratio, enzyme: total protein, was higher in well-fed rabbits, it was concluded that active synthesis of protein requires additional amounts of cathepsin II.

Zamecnik & Stephenson (138) used crude, thoroughly dialyzed cathepsin, which they activated by the addition of ultrafiltrates from normal and from hepatoma-bearing livers. Filtrates from the latter were considerably lower in cathepsin-activating ability; they were also low in glutathione. It would appear, therefore, that the presumably decreased proteolysis in malignant cells is due to the lack of activator, and not to the lack of enzyme protein, since the same authors had previously found increased amounts of the enzyme (139). In view of the finding that ultrafiltrates of fetal livers have a low glutathione content, but high cathepsin-activating ability, it was concluded that cathepsins may be activated by more than one tissue component.

Maver *et al.* (140) investigated catheptic activity in several types of primary and transplanted neoplasms in rats. No uniformity was found: catheptic activity was increased in some tumors, while no significant increase could be found in others.

As an extension of their studies of carbonyl group reagents as inhibitors for proteinases, Schales & Hill (141) have found that phenylhydrazine, hydrazine, hydroxylamine, dimedon, and semicarbazide inhibited different cathepsins of hog kidney to different degrees. Increasing quantities of cysteine decreased the inhibitory effect of phenylhydrazine, suggesting that both substances compete for the same group in the enzyme molecule.

The autolytic proteolysis (142) of mouse liver and muscle, and of yeast, was found to be affected by the presence of phosphates and phosphorolytic poisons. Krimsky & Racker (143) have shown that the inhibitor of glycolysis present in mouse brain has properties of cathepsin III. This finding emphasizes the significance of autolytic processes occurring in homogenates.

A cathepsin, activated by glutathione and ascorbic acid and having an optimum pH at about 5, has been found in the saliva and gastric juice of aphids (144).

Bacterial proteinases.—Jones, Stacey & Webb (145) studied the autolytic system of gram positive microorganisms. The first step in the process, which results in the loss of the staining properties, is essentially due to ribonuclease. The second step, involving the resolution of cytoskeletons rendered gram negative by the action of ribonuclease, is due to a proteolytic system, composed of two enzymes, one of which hydrolyzes casein but not peptone, and a second which hydrolyzes peptone exclusively.

The *K*-toxin of *Clostridium welchii* (collagenase) was further investigated by Bidwell (146). She found that either by exposure to pH 9 to 10 at low temperatures, or to a lower pH at 50°, true collagenase, an enzyme capable of disintegrating muscle and collagen paper, is destroyed. This treatment, however, releases another enzyme, which attacks hide powder and azocoll.

This second enzyme (gelatinase?) is destroyed either by heating to 60° for 10 min. or by exposure to a higher pH than 10.5.

Gorini & Fromageot (147) have described a very interesting proteinase from *M. lysodeikticus* which is completely inactive in the absence of calcium. The enzyme is not affected by sulfhydryl-group activators.

Virtanen & Winkler (148) have found that the essential proteolytic system of *E. coli* is not influenced by a pronounced decrease in the amount of nitrogen in the medium, while the adaptive enzymes are markedly decreased.

Smith & Worrel (149) have found that the action of the new antibiotic, chloramphenicol (chloromycetin), on gram negative bacteria cannot be explained as due to the inhibition of their proteolytic enzyme systems. The drug did not inhibit either bacterial proteinases or crystalline trypsin, chymotrypsin, pepsin, and papain.

Blood proteinases.—The appeal made three years ago by Fruton (1) for the adoption of a uniform terminology has not yet been realized.

No difference in the proteolytic activity of hemophilic and normal plasma was found (150). Fibrinolysin (plasmin), when incubated with vasopressin, destroyed it, but had no effect either on oxytocin or hypertensin (151). Mixing lung pulp with blood serum resulted in the appearance of proteolytic activity (152). Methods for the determination of proteolytic (153, 154) and antiproteolytic (155, 156) activities have been described, and both were clinically investigated (154, 157, 158, 159). The difference between staphylokinase and streptokinase activation of blood protease has been noticed (160, 161). In contrast to trypsin, the protease of plasma affected fibrin, but not fibrinogen (162). Heparin accelerated the autolysis of fibrin (163) when mixed with plasma, had no effect when mixed with serum, and inhibited fibrinolysis when used in the purified system (164). Loomis *et al.* (165) have described a method for the partial purification of antifibrinolysin. Methods for the determination of plasmin with a minimum of interference by the trypsin inhibitor of plasma, and vice versa, have been described (166). These were applied to a series of scarlet fever patients (167). The uncomplicated cases showed higher plasmin levels than inhibitor; in septic cases this difference was less pronounced. In cases complicated by rheumatic fever, inhibitor predominated over the amount of enzyme.

A method for the partial purification of fibrinolysin has been described (168). The complete activation of the enzyme was obtained only when treatment with chloroform was followed by an incubation period of several hours at 38°. This was interpreted as the formation of enzyme from pro-enzyme and not as the removal of anti-enzyme by chloroform.

Ungar & Mist (169) observed the formation of fibrinolysin (plasmin): (*a*) after the addition of specific antigen to serum from sensitized guinea pigs; and (*b*) after mixing normal guinea pig serum with peptone, agar, hyaluronic acid, chondroitin sulfuric acid, glycogen, pneumococcal polysaccharides, and heparin. They postulated that all these substances acted by

the removal of inhibitor. In contrast to streptokinase or chloroform, these substances functioned as activators only in the presence of an additionl thermolabile factor found in serum.

Urine proteinases.—In continuation of previous work Bucher & Anderson (170) investigated the output of uropepsin of cats under the influence of caffeine and histamine. No direct effect could be established, possibly because the output of uropepsin was influenced by appetite and the level of protein in the diet.

A claim has been made that the sex of the human fetus may be determined (171) before birth. The urine of women pregnant with male children contained a protease specifically splitting the protein prepared from the testes of rabbits, while women pregnant with female children excreted very little or no enzyme.

No protease capable of hydrolyzing the protein of the tubercle bacillus was found (172) in the urine of normal guinea pigs. However, the enzyme was claimed to be present in the urine of immunized animals and in the urine of tuberculous patients. In contradiction to a previous report (172), and to the claims of Abderhalden, no defensive enzymes were found (173) in the urine of immunized rabbits, but some proteolytic activity was always found in the urine of both immunized and control animals.

A protease, supposedly of the cathepsin type, has been reported in urine (174), but no experiments with specific substrates were made. The enzyme did not require activation by either cysteine or cyanide.

Proteolytic Enzymes and Protein Structure

The authority of Bergmann and the numerous advantages of using synthetic substrates for the investigation of specificity diverted attention from natural substrates for a period of several years. We are now witnessing a slow revival of the interest in natural substrates, probably due to (*a*) the development of chromatographic methods, and (*b*) the possible significance of peptides as growth factors. The knowledge gained by the enzymatic cleavage of proteins is obviously of basic significance in understanding their structure.

Proteolytic hydrolysis can be visualized in either of two ways: (*a*) as the progressive cleavage of the substrate molecule in halves until a final limiting length of peptide residue is reached beyond which cleavage no longer takes place; or (*b*), as a splitting off of peptides of constant length from the beginning to the end of the reaction.

In 1939 Tiselius & Eriksson-Quensel (175), on the basis of electrophoretic studies, concluded that the second type of reaction takes place when egg albumin is digested with pepsin. They called this type of cleavage the "all-or-none" type. This year the conclusion of Tiselius & Eriksson-Quensel was disproved in the home laboratory at Uppsala (176) and was confirmed at Boston (177).

An extensive study of the digestion of crystalline egg albumin by noncrystalline pepsin was presented by Moring-Claesson (176). Frontal analysis

on columns composed of carbon or aluminum hydroxide was used. Only part of the albumin molecules were attacked in the beginning of the reaction and some intact protein was still present at the end of the reaction. The cleavage products in the beginning consisted of deca or higher peptides, but at the end of the reaction tripeptides formed the major part of the products. Upon further fractionation of the peptide fraction, some dipeptides, but no free amino acids, were detected.

Beloff & Anfinsen (177) studied the products of proteolytic digestion of egg and serum albumins, γ-globulin, and fibrin. In all the experiments the undigested protein was precipitated and the peptide fraction analyzed for total and terminal amino nitrogen. The ratio of these two values indicated the number of amino acid residues per peptide. During peptic as well as during tryptic digestion, the value for this ratio was close to three, and was constant throughout the period of digestion. Such results would be expected from the "all-or-none" hypothesis of Tiselius & Eriksson-Quensel (175), but are contradictory to the findings of Moring-Claesson. Peptic digestion of fibrin showed comparatively large amounts of free amino acids. About 30 per cent of the total peptide bonds present in egg albumin were hydrolyzed by pepsin (177).

Egg albumin and serum albumin were digested for varying periods of time with pepsin (178). The digestion mixture was then subjected to electrophoresis. In addition to the undigested protein, nine other fractions were claimed to be present at pH 5.9: four were acidic, one neutral, and four basic.

Borsook *et al.* (179) have isolated a large peptide composed of at least 15 different amino acids: *peptide A*. This peptide was found in peptic digests of serum and egg albumins, γ-globulin, casein, fibrin, and insulin. The same peptide was isolated from intact livers of different animals, as well as from various other organs.

The proteolytic digestion of crystalline insulin has been the topic of several papers. In continuation of their previous work Butler *et al.* (180) investigated the digestibility of insulin by crystalline trypsin. Only a very small increase in nonprotein nitrogen was found as a result of enzyme action. The insulin which was recovered from the reaction mixture had, however, lost its potency. It was concluded that no more than one or two peptide bonds were split per unit of molecular weight 12,000.

Phillips (181) used crystalline α-chymotrypsin to digest insulin and attempted to characterize the resulting peptides by paper chromatography. No positive identification was possible, but it was estimated that five tyrosine and five phenylalanine residues remained in the undigested "core," while equal amounts of both amino acids were found combined as peptides or free in the digest.

Lens (182) investigated the action of carboxypeptidase on insulin. He concluded that certainly one and possibly three terminal amino acid residues of the insulin molecule are alanine. After three alanine residues were liberated at least six different amino acids appeared almost simultaneously. Shortening

of the insulin molecule by only a few amino acid residues resulted in a loss of potency.

Woolley (183) prepared dinitrophenyl insulin and dinitrophenyl trypsinogen, digested these with pancreatin, and isolated several crystalline yellow peptides, using the counter-current distribution technique. Four peptides were obtained from the digest of insulin and three from the digest of trypsinogen. Two of the peptides from insulin were indistinguishable from those of trypsinogen. They were composed of: (*a*) chromophore, glutamic acid, serine, threonine, alanine, leucine, isoleucine, and valine, and (*b*) the same plus aspartic acid. Partial hydrolysis of these peptides with acid led to smaller yellow compounds. Two of these were isolated and found to contain a chromophore and a residue of glutamic acid in common.

DeVerdier & Agren (184) studied tryptic digests of casein in an attempt to elucidate the structure of strepogenin. Pancreatin digested acetylated proteins to a smaller extent than natural proteins (185). Serum globulin irradiated with ultraviolet light was hydrolyzed by trypsin at a higher rate than the native protein (186). Trypsin was used to digest casein, edestin, ovalbumin, serum albumin, and capsular substance of *Bacillus anthracis*. The presence of a small number of gamma glutamic acid bonds was detected in the products of digestion (187). Ox lens capsule was resistant to the action of trypsin, but both normal and cataract lens were digestible (188). Raw and heated casein liberated equal quantities of amino acids (189) when digested with pepsin. Increasing the period of autoclaving (190) from 5 min. to 24 hr. caused a progressive decrease in the digestibility of pork by trypsin and by "erepsin" *in vitro*.

The proteolytic enzymes, including crystalline pepsin and α-chymotrypsin, have been used by Bowden & Peterson (191) for the release of biotin from natural sources. Their results strongly suggest that biotin may be attached to protein by a peptide linkage.

McLaren and co-workers investigated the action of ultraviolet light on pepsin (192) and ficin (193). The photochemistry of enzymes has been recently reviewed by McLaren (194).

Bresler and co-workers (195, 196) studied the reversal of proteolysis under high pressures ranging from 5,000 to 8,000 atm. Partially hydrolyzed proteins served as substrates. The products obtained, called "quasi-proteins," showed a pronounced similarity to the original proteins, but were not homogeneous.

Miscellaneous

Runnstrom and his group (197, 198) used crystalline trypsin and α-chymotrypsin for the investigation of the chemical nature of the vitelline membrane and the fertilization membrane of sea urchin egg. The former was readily attacked; the latter, which is partially derived from the former, was quite resistant to the action of proteolytic enzymes. The process of transformation of granular protein from an easily accessible into a more resistant

form was compared with the clotting of fibrin and the keratinization of epithelium.

Lundblad (199) has found that three minutes after fertilization, sea urchin eggs showed a very pronounced increase in proteolytic activity, which decreased after 30 min.

Proteolytic enzymes were found in the gastric juice of *Lamellibranchia* (200). No protease was present in the crystalline virus of *Bombyx mori* (201).

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NONOXIDATIVE, NONPROTEOLYTIC ENZYMES[1]

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Enzymes Acting on Phosphate Groups

Phosphatases.—While the number of reports on acid or alkaline phosphatase or adenosinetriphosphatase determinations has been impressive, one is entitled to reservations about their qualitative significance. For example, the measurement of adenosinetriphosphatase activity is usually performed by following the liberation of inorganic phosphate, by the usual histochemical or more quantitative techniques, from ATP in the presence of a tissue preparation. It is not always certain that the authors, particularly from disciplines other than enzymology, realize that they are following a complex series of events, probably mediated by, among others, the nonspecific phosphatases or pyrophosphatases, the nucleosidases, myokinase, nucleoside and nucleotide deaminases, as well as perhaps one or more true adenosinetriphosphatases (here used in the same sense as of the activity associated with myosin).

With the more general phosphatases, a similar degree of confusion prevails. A liberation of inorganic phosphate from various ester substrates by tissues, excreta, or secretions is ascribed to entities termed "acid phosphatase" or "alkaline phosphatase" depending on the pH which the investigator arbitrarily uses. This is in part because the measurement of serum phosphatase activity is relatively simple and sometimes meaningful to the clinician, and therefore the technique has been seized upon by many who are perhaps unaware of the existing evidence that there are more than two phosphatases in the body, or of other enzymes that might be studied. Gomori (1) concludes that there are indeed only two phosphatases, since the activity at pH 5 or pH 9 has the same cellular distribution regardless of which of many substrates was used. But, in a study of the enzymatic distribution by the Gomori technique, Newman *et al.* (2) have divided, on the basis of cellular distribution, the alkaline phosphatases into three classes, and each class obviously contains one or more nucleotidases or pyrophosphatases, so that distribution is not a very valid criterion. Further, using the hexosephosphates for substrates, Novikoff (3) has shown a class of phosphatases to be present in rat liver which have a slight action at pH 9, but a much higher activity at pH 7.4. These are in addition to preparations that are most highly active at lower or higher pH values. Swanson (4) presented evidence for the existence of a specific glucose-6-phosphatase in liver. Abul-Fadl & King (5) and Seligman & Manheimer (6) have confirmed earlier reports of the existence of at least two acid phosphatases in the erythrocyte, and the former workers have noted differences in the properties of both alkaline (7) and

[1] This review covers the period from November 1948 to October 1949.

acid (5) phosphatases from different organs which would be difficult to explain without invoking the presence of at least two different enzymes.

The following observations may be noted in passing: Modified analytical and histochemical methods have been reported (6,8,9). The occurrence of the phosphatases was studied in the rabbit genitourinary system (10), and in human neoplasms (11). Castration depletes the phosphatases of rat seminal vesicles, and the level is restored by androgens (12), but, on the other hand, estradiol causes an increase in the female genital tract (13). The serum alkaline phosphatase activity rises on low protein diets (14), but the acid phosphatase of muscle is raised by vitamin E deficiency, the alkaline phosphatase and adenosinetriphosphatase being unchanged (15). It is stated that biliary alkaline phosphatase is nothing more than excreted serum phosphatase (16), and contrariwise, that it is formed *de novo* and secreted by the liver (17). A study of the pH optimum of partially purified alkaline phosphatase has been made (18), with the finding that the value shifts toward the neutral range as the substrate concentration is lowered. An activation of alkaline phosphatase by a complex magnesium pyrophosphate ion was claimed (19). It has been noted (20) that under certain conditions cell components may give a false reaction for phosphatase activity in the Gomori histochemical technique. The previously claimed crystalline alkaline phosphatase of Roche's group has now been found to consist of protein adsorbed on a crystalline inorganic magnesium salt (21). Methods have been described for purification of intestinal and fecal alkaline phosphatases (7). The separation of organic factors, believed to be coenzymes, from alkaline phosphatases was reported by two groups (7, 22). Gomori (22a) made histochemical studies of the distribution of phosphamidase activity.

Among the inhibitor studies, several have borne on the activation by magnesium ions of the alkaline phosphatases from kidney or intestine. Abei (23) made a kinetic study of the inhibition by bicarbonate or ammonia buffers. The former acts competitively in the same manner as does orthophosphate, while the latter is noncompetitive. Possibly the ammonia forms a chelate, removing the metal activator. Abei interpreted his data to mean that magnesium acts as a stabilizer, perhaps by removing inorganic phosphate rather than as a true activator. An inhibition of alkaline phosphatases, but not acid phosphatases, by fluoro-substituted aromatics and by phthiacol (24) has also been interpreted as a removal of the metal activator by complex formation. Inhibitions by pyrophosphate and by diisopropylfluorophosphate may be caused similarly, although sulfate inhibition is more difficult to understand (25). Two groups (26, 27) have reported an inhibition of kidney and other alkaline phosphatases by beryllium ion. The inhibition begins at low concentrations (*ca.* 20 per cent at 1 to 3×10^{-6} *M*) but even at concentrations 1,000 times greater, the inhibition does not exceed 70 per cent. This fact makes the homogeneity of the enzyme activity suspect. The beryllium inhibition is interpreted as being a competition with magnesium (which would negate Abei's view given above). Magnesium addition partially over-

comes the beryllium inhibition but the data of the two papers are somewhat contradictory on this point.

Bodansky (28) has further investigated the amino acid inhibition of various phosphatases. Here again, differences in the behavior of the enzymes from different organs have been noted. Particularly, the activation of intestinal phosphatase by cobalt was markedly reduced in the presence of amino acids, while that of bone phosphatase was not. A striking result was the finding that the addition of cobalt and magnesium together is necessary for reactivation of amino acid inhibited bone phosphatase. This is interpreted as indicating the need for one of the transitional triad elements to "mediate" the activation by magnesium (it is interesting to compare this with the findings of Stickland with phosphoglucomutase discussed below).

Binkley (29) noted an inhibition of adenosinetriphosphatase by extracts of posterior pituitary containing the pressor principle, and an enhancement of the inhibition by acetylcholine, which is without action alone. The adenosinetriphosphatase activity of rat heart muscle homogenates is decreased by such agents as urethane, oxalate, fluoride, veratrine, etc., but is increased by cardiac glycosides (30). Zeller (31), in a study of the adenosinetriphosphatase from snake venoms, found the enzyme to be activated by Mg^{++}, Ca^{++}, Mn^{++}, Co^{++}, and CN^{-}, and inhibited by Zn^{++}, Cu^{++}, Hg^{++}, hydrogen sulfide, cysteine, ascorbate, diamines, and the anti-malarials.

In connection with his continuing studies of glycolysis, Meyerhof and collaborators have made interesting studies of the adenosinetriphosphatase question. Kielley & Meyerhof (32) have discovered a magnesium activated, particulate-bound adenosinetriphosphatase in muscle. A preparation free of myosin and actomyosin split one phosphate from ATP, had a pH optimum of 6.8, was inhibited by calcium ion, and had a Q-value of 8,000. The total yield was about equivalent to the myosin-adenosinetriphosphatase activity.

Even more interesting, perhaps, is the increasing volume of work on the adenosinetriphosphatase activities of other tissues. In general, the observations of the Meyerhof group divided the adenosinetriphosphatase activity into fractions associated with particulates (it is to be noted here that the tumor "soluble" enzyme may be particulate bound, and, if consistency of results is to be expected, probably is) and those which are soluble. In the case of the yeast enzyme, the implication is clear that solubilization of an adsorbed enzyme causes it to change its response toward such surface-active agents as octyl alcohol.

To summarize: in brain (33), most of the adenosinetriphosphatase activity is particulate-bound, and is inhibited slightly by decyl alcohol and less by octyl, but more strongly by azide. The lesser, soluble fraction is not inhibited, but is activated by octyl alcohol. In chick embryos (33) the activity is almost completely soluble, is activated by octyl alcohol, but inhibited 50 per cent by 0.02 M sodium azide, and somewhat by digitonin. In tumor homogenates (33) most of the activity is "soluble" (not

sedimentable in 10 min. at moderate speeds) and is inhibited by octyl alcohol. In dried yeast (34) adsorbed adenosinetriphosphatase is inhibited by octyl alcohol. Upon solubilization, the inhibition is lessened, and upon further purification, may become activating. Meyerhof states that this enzyme is a true adenosinetriphosphatase since it splits off one phosphate; he applies the name apyrase to those more indefinite systems which remove more than one phosphate.

In contrast, a Russian group reports (35) that most of the adenosinetriphosphatase of rabbit brain is soluble, activated by calcium but not by magnesium, and has an optimum at pH 7.6 to 8.0. This extract removes two phosphate groups from ATP. According to their report, the insoluble material contains some activity and is activated by both ions. Another group (36) reported that most of the adenosinetriphosphatase of normal and cancerous rat tissues occurs in the water-soluble fraction.

Steinbach, in addition to reporting adenosinetriphosphatase temperature coefficients for various vertebrate tissue extracts (37), reported that the soluble activity of muscle is inhibited by calcium ions, and less by magnesium (38), and that the insoluble activity is not influenced by either reagent until after extraction of the protein by high concentrations of potassium chloride; it then becomes calcium-activated in the typical myosin picture.

Krishnan (39) has achieved a 1,000 to 2,000-fold purification of what appears to be a true apyrase from potato. Although the enzyme, which is calcium activated, still contains nucleotidases, phosphatases, and perhaps a nonspecific pyrophosphatase, in dilute solutions it only splits two phosphates from ATP.

In a rare type of fundamental study, Cohn investigated the mechanism of phosphatase and phosphorylase activity (40) by use of O^{18}. She found that in the nonenzymatic acid hydrolysis of glucose-1-phosphate the C-O bond was ruptured. This same bond is split by muscle and sucrose phosphorylases acting in either direction in the reaction. However, the prostatic acid or intestinal alkaline phosphatases split the O-P bond, presumably in a mechanism similar to nonenzymatic alkaline hydrolysis.

Meyerhof & Green used intestinal alkaline phosphatase and prostatic acid phosphatase as tools for determining the equilibrium constants, and therefore the free energy changes, for the hydrolysis of various hexose and glycerol phosphate esters (41). The $\Delta F°$ values obtained were (in calories) glucose-6-phosphate, $-3{,}000$; galactose-6-phosphate, $-3{,}000$; mannose-6-phosphate, $-2{,}650$; fructose-1-phosphate, $-2{,}790$; α-glycerophosphate, $-2{,}200$. All were calculated at pH 8.5 and at 38°. At pH 5.8 the values were about 400 calories less. Values were given for equilibrium constants of other compounds. Two interesting points were made. The equilibrium constants for these hydrolyses cannot be determined from the kinetics of the separate forward and back reactions, since the reaction is inhibited by phosphate. Further, the speed of the reverse reaction is increased greatly by added

organic phosphate of a higher energy content. Seemingly, the effect may depend upon the amount of energy in the phosphate bond of the added substance, since phosphocreatine is more active than is glucose- or fructose-1-phosphate. The same authors investigated this effect further (42), using isotopic phosphorus in a manner similar to that employed by Axelrod (see below), and found that the acceleration of the reverse reaction is due to a transfer of phosphate from the higher-energy component, and that this transfer is not mediated via inorganic phosphate, as Axelrod also showed for citrus fruit phosphatase. In other words, in the presence of phosphatases, a phosphate acceptor such as glycerol, and inorganic phosphate, and under circumstances such that the concentration of the components, especially water, favors reversal of hydrolysis, the formation of the ester phosphate is accelerated by the presence of a phosphate of higher energy, and the extra phosphate thus formed is derived from the higher energy compound directly.

Axelrod's experiments (43) involved the incubation, in the presence of a citrus fruit enzyme, termed phosphotransferase (although the work of Meyerhof & Green would indicate it is probably an ordinary phosphatase), of *p*-nitrophenylphosphate and methanol. The products were *p*-nitrophenol and methylphosphate. With the aid of isotopic phosphorus, it was established that this transfer occurs without the phosphate passing through the inorganic stage.

Another phosphatase of interest which has been reported by Feinstein & Volk (44), hydrolyzes phosphate from phosphoproteins without removing nitrogen. The enzyme, found in various organs of the rat, and in the spleen of several animals tested, is activated by some bivalent ions (Mg^{++}, Mn^{++}, Ba^{++}, Ca^{++}) in the absence of reducing agents, but not in their presence. Such ions as K^{+} and Zn^{++}, and those of the transition triad, Fe^{++}, Co^{++}, and Ni^{++}, have no effect.

Phosphorylase.—New preparations have been reported (45, 46). Phosphorylase-*a* is claimed to have mixed phosphorylated adenosine and inosine compounds as prosthetic groups (47). Hestrin (48) studied the limit dextrins formed by β-amylase and muscle phosphorylase. He found that phosphorylase would not attack the dextrin of β-amylase, but that the latter would still hydrolyze the phosphorylase dextrin to the extent of 24 per cent, roughly one mole of maltose being liberated per end group of dextrin. Phosphorylase priming action was still exerted by the phosphorylase dextrin, but not by that of the β-amylase. Calculations were made for the following equilibrium:

$$(\text{glucose})_n + \text{glucose-1-phosphate} \rightleftharpoons (\text{glucose})_{n+1} + H_3PO_4$$

where $(\text{glucose})_n$ is the concentration of the end groups of the priming polysaccharide. A K of 1.3 to 1.5 was found over a four-fold range of primer concentration.

Lundbaek & Goranson (48a) studied the phosphorylase activity of muscle during fasting. The total activity was found to increase and could again be diminished by refeeding. The relative proportion of phosphorylase-*a* was

also found to increase, and these authors suggest the effect may serve as a mechanism for regulating the muscle glycogen stores during starvation.

Phosphoglucomutase.—There has been a considerable upsurge of interest in this enzyme during the past year, mostly centering on the action of glucose-1,6-diphosphate as a coenzyme, first announced by Leloir's group (49, 50, 51) and then confirmed by Sutherland *et al.* (52, 53). The mode of action is as follows:

$$\text{glucose}^a\text{-1-phosphate} + \text{glucose}^b\text{-1,6-diphosphate} \rightarrow$$
$$\text{glucose}^a\text{-1,6-diphosphate} + \text{glucose}^b\text{-6-phosphate}$$

The molecules are here labelled "a" and "b" to emphasize that the original substrate molecule now becomes the coenzyme and then can react with another glucose-1-phosphate molecule which in turn becomes the coenzyme, etc. The coenzyme has been shown to be formed by a yeast or muscle preparation in the presence of ATP and glucose-1-phosphate (51). This new kinase (presumably) has a pH optimum of 6.8 and is activated by Mg^{++} and Mn^{++}.

Stickland, however, disputes the view that muscle phosphoglucomutase has the glucose diphosphate as coenzyme (54), and believes that the true coenzyme is fructofuranose-1,6-diphosphate. While he concedes that his preparation of the latter compound also contains the glucose derivative, he bases his claim on a differential destruction of the glucose compound and upon a separation of coenzyme activities toward the yeast and the muscle enzymes. The implication is plain in the paper of Sutherland *et al.* (53) that the glucose compound is the coenzyme for both the yeast and the muscle enzyme. A further complication is introduced by the findings of Jagannathan & Luck with 50-fold purified enzyme from muscle (55, 55a). They found enzyme-bound phosphorus in a very labile form, which exchanges with the phosphate of glucose-1-phosphate during the formation of glucose-6-phosphate.

Another discrepancy is found in the report by Stickland (56) that at low dilutions of the enzyme, Al^{+++}, Pb^{++}, Cr^{+++}, Fe^{+++}, or Ce^{+++} are required in addition to Mg^{++} for full activity, and that Co^{++} or Mn^{++} cannot replace Mg^{++}. 10^{-5} *M* Cr^{+++} gives maximum activity. Sutherland, on the other hand, states (57) that the enzyme, whether derived from skeletal muscle, heart muscle, or yeast, is activated by metal-binding agents, e.g., cysteine, 8-hydroxyquinoline, histidine, or another protein. Cysteine, when permitted to stand in contact with the enzyme prior to incubation, is inhibitory by virtue of its reducing action. Jagannathan & Luck (55) had earlier reported the activating action of a protecting protein or cysteine, and they also found added sodium sulfite to be required for full activity. The increased activity observed in the presence of an optimum concentration of albumin in the presence of sulfite suggests that the latter may act to prevent reduction of the enzyme. Curiously, the addition of sulfite raised the activity about 50 per cent in the presence of 0.0005 Mn^{++} and nearly three-fold in the presence of 0.0005 *M* Mn^{++} plus 0.005 *M* Mg^{++}, although without sulfite the activities were identical with both metals; it is presumed by the reviewers that the

concentration given represents the optimum for Mn^{++}, since 0.005 *M* is stated to be the optimum for Mg^{++}. Co^{++} was also found to be activating to an extent nearly equivalent to that of Mn^{++}. Ni^{++} was without effect, while K^+, Ca^{++}, Hg^{++}, Cu^{++}, and arsenate were all inhibitory. Obviously, more studies are needed to explain these apparent discrepancies.

Hexokinase.—Both Meyerhof & Wilson (58) and Wiebelhaus & Lardy (59) have presented evidence for the existence of separate gluco- and fructokinases in animal tissues, in contrast to the nonspecific yeast enzyme. Meyerhof & Wilson cite the differing ratios of activity of different tumor preparations toward the two substrates. Wiebelhaus & Lardy noted the same with different brain preparations, and also a differential inhibition by sodium ions. The brain preparation contains both hexokinase and phosphohexokinase in soluble form. It phosphorylates fructose, glucose, mannose, and their corresponding 6-phosphates, but not galactose, ribose, or gluconic acid. The K_s for glucose (with 0.002 *M* ATP) is less than 10^{-4} *M*, while that of fructose is 0.0014 *M*.

Vestling *et al.* (60) have apparently unequivocal evidence for a fructokinase in liver, in that fructose is rapidly glycolyzed but glucose is inert.

In a study by Christensen, Plimpton & Ball with hemolyzed rat erythrocytes (61), relative rates of 1, 0.77, and 0.36 were found with glucose, mannose, and fructose. Galactose was not phosphorylated. They noted an inhibition of the hexokinase by some plasma factor. This factor was not affected by insulin or by adrenocortical extracts from normal or from diabetic or hypophysectomized animals. Neither diabetes nor hypophysectomy had any effect on the enzyme level. Stadie & Haugaard (62) have presented further evidence for the nonparticipation of insulin in the hexokinase reaction, by showing that adrenocortical extracts or insulin are without effect on the hexokinase reaction in muscle preparations from alloxan diabetic rats, and again no difference was observed in the level of activity compared to control animals.

Griffiths (63) reports a 100 per cent inhibition of muscle hexokinase by 5×10^{-3} *M* alloxan, and a 70 per cent inhibition of phosphohexokinase at a 2.9×10^{-3} *M* concentration. These results may have some bearing on observations of hexokinase levels in alloxan diabetic animals.

Phosphoglycericmutase.—Sutherland, Posternak & Cori (52) have shown that 2, 3-diphosphoglyceric acid, the significance of which has been unknown since its discovery by Greenwald, is the coenzyme for this enzyme, acting analogously to glucose-1,6-diphosphate in the phosphoglucomutase reaction.

Phosphopyruvate-ATP transphosphorylase.—Meyerhof & Oesper (64) made a study of the phosphopyruvate-ATP equilibrium, using an enzyme partially purified from rabbit muscle. The equilibrium constant for the reaction:

$$\text{phosphopyruvate} + \text{ADP} \rightleftarrows \text{pyruvate} + \text{ATP}$$

was determined to be of the order of 2,000 at 30°. The pH of the reaction

mixture, containing ammonium sulfate and bicarbonate in unspecified concentrations, was not given. However, it is clear that the $\Delta F°$ for the reaction is of the order of $-4{,}000$ calories and that the amount of phosphopyruvate which can coexist with adenosinediphosphate (ADP) is very small. In the same paper, the equilibrium constants for the enolase and phosphoglyceric mutase reactions have been redetermined to be 2.9 and 6 respectively.

Diphosphopyridine nucleotide (DPN)-cleaving enzymes.—Kornberg has continued his studies on DPN metabolism. He found (65) an enzyme in yeast which cleaves DPN in the presence of pyrophosphate according to the following:

$$\text{DPN} + H_4P_2O_7 \leftrightarrows \text{Nicotinamide-nucleotide} + \text{ATP},$$

the equilibrium constant being 2 to 3 as written. Triphosphopyridine nucleotide (TPN), flavin adenine dinucleotide (FAD), and pyrophosphate were not attacked in his system, but reduced DPN was also cleaved. It is suggested that both the formation of pyrophosphate and of DPN can be accounted for by this reversible reaction.

Kornberg & Lindberg (66) found two types of DPN cleavage in animal tissues. In rabbit brain, DPN is attacked via a nucleosidase type reaction, since the nicotinamide-ribose bond disappears at the same rate as DPN. This is the activity inhibited by nicotinamide. In the particulate matter of rabbit kidney, cleavage to nicotinamide-nucleotide and adenylic acid occurs through pyrophosphatase action.

Myokinase.—Kotel'nikova (67) has found myokinase to be present in liver, heart, kidneys, and erythrocytes in as high concentrations as in muscle. Hydrochloric acid treatment, according to this author, destroys adenosinediphosphatase activity (adenosinetriphosphatase + myokinase?), but not myokinase. Boiling in acid for a short time was without effect, but destruction ensued in a neutral medium.

Hydrolytic Enzymes affecting other than Phosphate Linkages

Carbohydrases.—Schwimmer & Balls (68) have isolated and crystallized the α-amylase of germinated barley (malt). The enzyme was found to have a molecular weight of $59{,}500 \pm 900$ and a turnover number of 19,000. Calcium appears to be required for activity. The adsorption of α-amylase on starch and starch derivatives has been investigated by the same workers (69). Lane & Williams (70) observed that the inhibition of pancreatic α-amylase by γ-hexachlorocyclohexane is overcome by inositol. On this basis, these authors suggest that inositol is an active constitutent of this enzyme. The action of α- and β-amylases on glycogen and on partly acid-hydrolyzed glycogen has been investigated by Carlqvist (71). Alfin & Caldwell (75) have studied the action of a maltase-free pancreatic amylase preparation on unfractionated potato starch, lintner soluble potato starch, and straight-chain fractions from corn starch.

Lineweaver *et al.* (72) have succeeded in highly purifying a polygalac-

turonase preparation. It failed to hydrolyze methyl-α-D-galacturonide which thus differentiates it from the simple glycosidases. Ingleman (73) found that extracts of *Cellvibrio fulva* cultures can enzymatically break down dextrans to large fragments without formation of glucose or disaccharides. A modified Willstatter method, using dinitrosalicylic acid, for the rapid determination of amylase is reported by Smith & Stocker (74).

Glucuronidase.—The presence of a third β-glucuronidase in aqueous extracts of ox spleen was reported by Mills & Paul (76). This enzyme has a pH optimum of 3.4 as compared with those of the two previously reported glucuronidases (77) of 4.5 and 5.2. The enzymes with pH optima of 3.4 and 4.5 were found to be inhibited by polycarboxylic acids and polyhydroxymono- and dicarboxylic acids. Saccharic acid proved to be the most powerful inhibitor, thus confirming the report of Karunairatnam & Levvy (78). The latter investigators first reported that saccharic acid competitively inhibited the hydrolysis of phenylglucuronide by mouse liver or kidney β-glucuronidase preparations. Saccharic acid, on the other hand, was without influence on the synthesis of *o*-aminophenylglucuronide by mouse liver slices, thus indicating the nonidentity of the hydrolytic and the synthetic enzyme systems. Levvy & Storey (79) have developed a colorimetric method for *o*-aminophenylglucuronide suitable for measuring the enzymatic synthesis of this compound by tissue slice preparations.

The presence of two β-glucuronidases in mouse liver and spleen was reported by Kerr *et al.* (80) in confirmation of Mills' findings referred to above for ox spleen. Both glucuronidase fractions responded identically to agents which cause changes in the enzyme activity. Ascorbic acid in a concentration of 20 mg. per cent was found by Becker & Friedenwald (81) to inhibit liver glucuronidase up to 90 per cent. Heparin and impure hyaluronic acid inhibited an ammonium sulfate precipitated glucuronidase preparation up to 50 per cent.

Hyaluronidase.—The literature on hyaluronidase continues to be filled with many papers which report studies using ill-defined enzyme and substrate preparations, in addition to questionable methods of assay. There is great need for further enzyme purification, synthetic or better defined substrates and more reliable analytical methods. In particular there is need for additional studies on the nature and extent of cleavage of the substrates now employed and differentiation between the depolymerizing action on the one hand and the subsequent cleavage on the other. In this connection the excellent papers by Hahn (82) which pertain to the nature of the hyaluronic acid splitting enzymes and the end products should be brought to the reader's attention, since they apparently have escaped previous review in the *Annual Review of Biochemistry.*

Freeman *et al.* (83) have effected an 80-fold purification of bovine testis hyaluronidase by means of ethanol and ammonium sulfate fractionation. Byers *et al.* (84) described a method for the preparation of polymerized hyaluronate in high yield from umbilical cord. A study of conditions for

stabilization and storage of testicular hyaluronidase was carried out by McCullagh *et al.* (85). Werle *et al.* (86) have studied the effect of various salts and organic compounds on the viscosity-lowering activity of partially purified hyaluronidase preparations.

Evidence has been presented (87) to show that hyaluronidase is formed at an early stage of spermatogenesis, the enzyme being present in spermatocytes, and a close correlation between spermatogenic activity and hyaluronidase content of bull testis has been reported (88). A similar correlation was observed in the case of humans (89). Further studies on the factors influencing the liberation of hyaluronidase from testis homogenates and spermatozoa have been reported by Perlman *et al.* (90).

Lundquist (91) has made a study of hyaluronic acid depolymerization by hyaluronidase and found that the reaction obeyed the kinetics of a monomolecular reaction. The reaction constant under specified conditions can be used as a measure of hyaluronidase activity. A modified turbidimetric method for assay of hyaluronidase has been described by Warren *et al.* (92). Fulton *et al.* (93) have devised a method suitable for the quantitative assay of small quantities of hyaluronidase or hyaluronidase inhibitor. According to Freeman *et al.* (94) magnesium ion is essential for hyaluronidase inhibitor activity of human blood. Wattenberg & Glick (95) studied a large series of compounds relative to their inhibitory effect on hyaluronidase. Of this large series it was found that sterol conjugates, hemoglobin derivatives, and bile were active as inhibitors. However, these compounds do not account for the inhibitory effect of serum. On the other hand, it was found that stercobilin, bilirubin, protoporphyin, hemin, and the bile salts prevented the inhibiting effect of serum. Bile and urine were the only body fluids, in addition to serum, which were found to inhibit hyaluronidase.

Cholinesterase.—The problem of defining the specificity of the serum and tissue choline esterases continues with some progress being made. It might have been predicted that a simple distinction between so-called "true" and "pseudo" cholinesterases would not stand in the face of newer substrate analogues and inhibitors. Those interested in the role of acetylcholine breakdown as an integral step in nerve conduction appear determined to assign a narrow specificity to such an esterase. Augustinsson & Nachmansohn (96) have proposed the term acetylcholine-esterase for an enzyme found in nerve and muscle tissue and erythrocytes which has the following characteristics: (*a*) a high affinity for acetylcholine; (*b*) no other ester yet tested is split at a higher rate; (*c*) propionylcholine is hydrolyzed at the same or a lower rate, while butyrylcholine is split at a lower rate; (*d*) noncholine esters are not split at all or at low rates; (*e*) a high turnover number. On the basis of these and other suggested properties, these investigators feel that acetylcholine-esterase can be distinguished from other esterases, including nonspecific cholinesterases such as those found in serum and pancreas. An extensive study of human erythrocyte cholinesterase has been published by Adams (97). The general impression that this is a highly specific enzyme is challenged

by the findings that this preparation is capable of hydrolyzing a large number of noncholine esters. In general it was found that the more closely the alcohol group of a given ester simulated the choline configuration the more rapidly is it hydrolyzed. Of the substrates investigated, 3,3-dimethyl-butyl-acetate, the carbon analogue of acetylcholine, was found to be most rapidly hydrolyzed next to acetylcholine itself. In comparing the hydrolysis rates of the substituent acid groups of choline and noncholine esters it was found that all acetates investigated were hydrolyzed; the corresponding propionates and formates were less readily hydrolyzed, and butyrates slowly or not at all. This author rejects the distinction between choline esterases on the basis of substrate specificity alone.

Studies by Adams & Whittaker dealing with the properties of esterases in human plasma (98) and the specificity of plasma enzyme and its relation to erythrocyte cholinesterase (99) indicate the following distinguishing features between the plasma and erythrocyte enzymes: (*a*) for any given alcoholic group the optimal acyl group for the erythrocyte enzyme is acetate and for the plasma enzyme is butyrate; (*b*) both enzymes hydrolyze most rapidly those aliphatic esters which approach most closely the choline configuration, but differ with respect to the effect of chain branching in the carbon atoms of the alcohol adjacent to the ester link; and (*c*) the enzymes differ in their kinetic behavior and in the extent to which they are inhibited. The chief difference therefore, according to these authors, between the plasma and erythrocyte enzymes is not primarily one of specificity range but rather that the acyl group which is optimal for the erythrocyte enzyme differs from that which is optimal for the plasma enzyme. This viewpoint is not far different from that maintained by Augustinsson & Nachmansohn. Augustinsson (100) studied the effect of substrate concentration on the different choline-ester splitting enzymes. He found that whereas the serum enzymes gave the usual type of dissociation curve, the esterases from a wide variety of conductive tissues studied and from erythrocytes showed a sharp optimum acetylcholine concentration. McNaughton & Zeller (101) and Zeller *et al.* (102) have suggested the use of ethylchloroacetate plus eserine and β-chloroethylacetate as substrates for distinguishing the so-called "true" or "specific" esterases found in erythrocytes and snake venom from the "pseudo" or "non-specific" found in plasma. However, Meyers & Mendel (103) maintain that the eserine insensitivity toward ethylchloroacetate is due to the eserine insensitive aliesterases, rather than cholinesterases, in the preparations of McNaughton & Zeller. Later studies from the same laboratory by Zeller *et al.* (104) indicate that cholinesterase from human erythrocytes and snake venoms is capable of hydrolyzing ethoxyethanol acetate, desoxycorticosterone acetate, ketopropanol acetate, phenyl acetate, *p*-nitrophenyl acetate and acetyl salicylate.

Bovet-Nitti (105) studied the hydrolysis of a series of aliphatic and aromatic choline and triethylethanolamine esters by "pseudo" and "true" cholinesterases.

A detailed study of the inhibiting effect of diisopropyl fluorophosphate, tetraethyl pyrophosphate, prostigmine, and eserine on purified acetylcholine esterase prepared from the electric organ of *Electrophorus electricus* has been reported by Augustinsson & Nachmansohn (106). Tetraethyl pyrophosphate was found to be most potent, causing almost immediate irreversible inhibition. The simultaneous addition of acetylcholine with each of the inhibitors led to some protective action which the authors interpret as suggesting that a common active center on the enzyme is involved in the effect by the four different inhibitors.

Hawkins & Mendel (107) found that the N-*p*-chlorophenyl-N-methylcarbamate of *m*-hydroxyphenyltrimethyl-ammonium bromide, a prostigmine analogue, was a specific inhibitor of "true" cholinesterase. The inhibitory effect of physostigmine (eserine) on rat brain cholinesterase using acetylcholine and acetyl-β-methylcholine was studied by Cohen *et al.* (108). Some differences in the degree of inhibition with the two substrates were observed.

Schaefer & Maier (109) have carried out a detailed study on the measurement of cholinesterase activity of blood.

The cholinesterase activity of rat plasma ("pseudo" or "nonspecific") was found to be independent of the "true" (or "specific") cholinesterase activity of tissues by Mendel *et al.* (110). According to Little, both dog (111) and mouse (112) brain homogenates can be separated into two "specific" cholinesterase fractions by centrifugation. Serum cholinesterase levels have been studied in various pathological conditions by Levine (113), Gitman *et al.* (114) and Hawkins *et al.* (115). The cholinesterase activity of cerebrospinal fluid was found by Tower and McEachern (116) to be chiefly of the "specific" or "true" type. While no significant changes in cholinesterase activity of cerebrospinal fluid was noted in cases of epilepsy (117), there was a decrease in cases of craniocerebral trauma (118).

A histochemical method for localizing cholinesterase activity using acetylthiocholine as substrate is reported by Koelle & Friedenwald (119).

An extensive study of the distribution and specificity of cholinesterase in venoms from different species of snakes is reported by Zeller (120) and Zeller & Utz (121). The cholinesterase activity of various portions of elephant brain was investigated by Zeller (122). Rabbit polymorphonuclear leukocytes have been shown to have esterase, but not cholinesterase, activity (123).

Potentiometric methods for determining cholinesterase activity are described by Delaunois & Casier (124) and Michel (125).

Hestrin (126) has determined the equilibrium constant for the acetylcholine acetylcholine -esterase system and obtained a value of 0.25 at 23° with a $\Delta F°$ value of the order of $^{-}3{,}100$ cal. Under suitable conditions, synthesis of acetylcholine from choline acetate could be demonstrated. Incubation of the enzyme with hydroxylamine and acetate or propionate led to the formation of hydroxamic acid. Thus acetylcholine -esterase is able to mediate both nitrogen- and oxygen-acylation.

Cholesterol esterase.—Nieft & Deuel (127) have found that saline extracts of rat liver and intestine are capable of both hydrolyzing and synthesizing cholesterol esters. The hydrolytic activity is increased by the addition of soybean lecithin; the synthetic process requires phosphate ions. The two effects are apparently catalyzed by independent enzyme systems. Further study of the hydrolytic enzyme by Nieft (128) revealed it to consist of at least two components, a heat-labile globulin and a heat stable nondialyzable factor. The preparation of a cholesterol oleate mixture, suitable as substrate for the assay of cholesterol esterase of tissues and fluids, is reported by Yamamoto *et al.* (129).

Other esterases and lipases.—The hydrolysis of acetyl derivatives of morphine by rabbit and guinea pig plasma and liver was studied by Ellis (130). Tributyrinase appears to be the enzyme responsible for the hydrolysis of these compounds. According to Fodor (131), the lipolytic enzymes of insects are of two types, one of which splits esters of short chain and the other of long chain length. Castor bean lipase is inhibited approximately 70 per cent by 3.03×10^{-3} *M* 2,4-dichlorophenoxy acetic acid (132). Tuba & Hoare (133) found that serum lipase levels of rats made diabetic with alloxan are elevated to 40 to 45 per cent above normal. The elevation is most pronounced seven days after the injection, and persists up to three months.

Arginase and amidases.—Mohamed (134) has repeated the procedure which, according to Bach (135), yields crystalline arginase. A heterogenous mixture of proteins resulted. The round structures which Bach considered to be crystals proved to be not only noncrystalline but also free of arginase activity. A detailed study of the arginase activity of liver and mammary gland arginase has been conducted by Folley & Greenbaum (136).

The nature of the amidases which hydrolyze aromatic and aliphatic amides has been investigated by Bray *et al.* (137). These authors report that the aromatic amides are hydrolyzed by enzymes other than those which hydrolyze glutamine and asparagine. Workers from the same laboratory (138) have found that long term incubation experiments using rabbit liver, kidney, and brain glutaminase preparations result in the nonenzymatic formation of pyrollidone carboxylic acid as an end product of glutamine hydrolysis. Errera (139) succeeded in separating two glutaminases from rat liver by means of high speed centrifugation. Glutaminase I, which is found largely in the sediment, is activated by phosphate but not by pyruvate and is apparently bound to the insoluble particles; glutaminase II is water soluble and is active only in the presence of α-keto acids. The distribution and phosphate activation of the glutaminases in different tissues of different species have been investigated by Errera & Greenstein (140). The properties of asparaginases prepared from rat liver extracts was reported by Greenstein & Price (141). Mylon & Heller (142) found that the glutaminase activity in slices from frozen dog or cat kidney cortex was greater than in extracts or homogenates of the same organ. The activity in slices was found to be markedly stimulated by pyruvate and phosphate. The greatest stimulation, however, resulted from the addition of phosphate plus β-hydroxybutyric acid.

The possible significance of these findings with relation to the production of urinary ammonia is discussed by these authors.

Urease.—Kistiakowsky & Lumry (143) have studied the temperature coefficients of the hydrolysis of urea by urease. In the absence of strong oxidizing agents and sulfite ions the Arrhenius plot was a straight line with an activation energy of 8,830 cal. at all temperatures studied. Deviations from linearity of the Arrhenius curve in the presence of sulfite ions, according to the authors, are due to reversible inhibition of the enzyme. Laidler & Hoare (144) carried out a kinetic study of urea hydrolysis by urease and found evidence for inhibition during the course of hydrolysis by the ammonium ion. The authors offer a mechanism to explain the observed decrease in reaction rate at high substrate concentrations. Desnuelle & Rovery (145) reported that urease is not inactivated when its "reactive"-SH groups are blocked with phenylisocyanate. However, inactivation follows when the "nonreactive"-SH groups are blocked.

Cystathionase.—The crystallization of cystathionase from rat and pig liver has been reported by Binkley (146). An alcohol soluble coenzyme, magnesium, and inorganic phosphate are required for activity. While crude liver preparations gave rise to cysteine as an end product, this was not a primary product with the purified enzyme. The exact nature of the cleavage products with the purified enzyme thus remains to be established. Carroll *et al.* (147) found that rat liver preparations cleaved cystathionine anerobically to form cysteine and β-ketobutyric acid under similar incubation conditions. DL-Homoserine also formed β-ketobutyric acid. It thus seems possible that homoserine is an intermediate in the cleavage of cystathionine.

Carboxylases

Carbonic anhydrase.—An excellent review by Van Goor (185 references) of this subject has recently appeared (148). The use of this enzyme as a tool in studying the mechanism of reactions involving H_2CO_3, CO_2 or HCO_3^- is described by Krebs & Roughton (149). These authors describe the use of Warburg manometers for estimation of carbonic anhydrase activity. Altschule & Lewis (150) have also reported a technique using the Warburg apparatus for estimating carbonic anhydrase activity of blood. The inhibitory effect of 25 different sulfonamides of carbonic anhydrase was studied by Krebs (151).

α-Keto acid carboxylases.—Vennesland *et al.* (152) have made a detailed study of the preparation and properties of oxaloacetic carboxylase in plants. The enzyme, which was found to be associated with a TPN linked malic dehydrogenase was shown to be widely distributed in the roots, tubers, leaves, and seeds of higher plants (153). The carboxylation of α-ketoglutaric acid by a parsley root extract was demonstrated by Ceithaml & Vennesland (154). Mn^{++} or Co^{++} was found to be necessary. In the presence of TPN the initial fixation product is reduced to isocitric acid. Plaut & Lardy (155) purified an oxaloacetic decarboxylase from *Azotobacter vinelandii* 40 to 50-

fold. The enzyme was activated by Mn^{++}, Co^{++}, Zn^{++} and slightly by Mg^{++}. No fixation of carbon dioxide into oxaloacetate could be demonstrated with this enzyme system. The influence of cations on the decarboxylation of oxaloacetic acid in the presence and absence of oxaloacetic decarboxylase was studied by Speck (156). Mn^{++} was found to be the most effective.

The synthesis of thiamine triphosphate and its ability to function as codecarboxylase is reported by two groups of investigators. Viscontini *et al.* (157) found the compound to be equally as active as cocarboxylase, while Velluz *et al.* (158) reported it to be only one-fourth as active. The latter group also found that while the compound was hydrolyzed by potato apyrase, pig muscle adenosinetriphosphatase was inactive.

Amino acid decarboxylases.—The claim by Karrer & Viscontini (159) that pyridoxal-3-phosphate is the coenzyme for amino acid decarboxylases is refuted by Umbreit & Gunsalus (160). The latter investigators compared their preparation of pyridoxal phosphate (161) (position of the phosphate group unspecified but known not to be in the 3 position) with a sample of pyridoxal-3-phosphate sent them by Karrer & Viscontini. The Umbreit & Gunsalus preparation proved to be 2,000 to 3,000 times more active using the tyrosine decarboxylase system from *Streptococcus faecalis R.* Sloane-Stanley (162) found that tyrosine decarboxylase perparations from *S. faecalis R.* decarboxylate *m*-hydroxyphenylalanine at about 30 per cent of the rate at which tyrosine is decarboxylated. *o*-Hydroxyphenylalanine and 2,5-dihydroxyphenylalanine were not decarboxylated. Blaschko (163), on the other hand, reported that extracts of rat liver and guinea pig kidney decarboxylate DL-*o*-hydroxyphenylalanine. The enzyme responsible in animal tissues is in all probability the dopadecarboxylase system. Of interest was the finding of Blaschko that DL-*o*-hydroxyphenylalanine was oxidized by both the D-amino acid oxidase of pig kidney and the L-amino acid oxidase of cobra venom. A detailed study on the inhibitory effect of pyridoxine and other compounds on the histidine decarboxylase from bacteria and animal tissues was carried out by Werle & Koch (164). The authors propose a scheme to explain the mechanism of amino acid decarboxylation involving an intermediate Schiff's base formed between pyridoxal phosphate and the α-amino acid. Martin *et al.* (165) have observed an inhibition of histidine decarboxylase by flavanoid compounds, particularly quercitin and *d*-catechin.

Miscellaneous

Nucleases.—A desoxypentose nuclease and its specific inhibitor have been isolated from yeast by Zamenhof & Chargaff (166). The inhibitor is stated to be specific for yeast desoxypentosenuclease. A comparison of the properties of the yeast and pancreatic enzymes revealed differences in solubility and pH optima. Dabrowska *et al.* (167) have isolated a protein fraction from pigeon crop gland which inhibits pancreatic desoxyribonuclease. While this preparation was not tested on yeast nuclease, the inhibitor is probably not identical with that of Zamenhof & Chargaff. The nature of the cleavage

products of yeast ribonucleic acid after incubation with ribonuclease was studied by Bacher & Allen (168). Weissman & Fisher (169) have found that Mg^{++} alone causes a marked decrease in the relative viscosity of sodium thymonucleate solutions. Friedkin *et al.* (170) demonstrated the enzymatic synthesis of hypoxanthine desoxyriboside from hypoxanthine and desoxyribose phosphate in the presence of liver nucleoside phosphorylase.

Lysozyme.—Inhibition of lysozyme by detergents and other compounds was studied by Smith & Stocker (171) and Alpert & Martin (172). The inactivation of lysozyme by iodine and reactivation by sulfite and thioglycol has been reported by Fraenkel-Conrat (173). Proctor & Dickman (174) described a turbidometric method for lysozyme assay.

Aldolase.—Sibley & Lehninger (175) have described a simplified chemical procedure for the estimation of aldolase and have estimated the activity of this enzyme in a large series of normal and neoplastic tissues. In confirmation of earlier studies by Warburg & Christian (176), these authors report the presence of aldolase in normal serum with an increase in conditions of neoplasia (177). While the serum aldolase content of tumor-bearing rats was markedly and uniformly high, in human cancer the serum aldolase was elevated in only about 20 per cent of the cases.

Baranowski & Niederland (178), redetermined the turnover number of crystalline rabbit muscle aldolase. The new values obtained are 2,500 and 4,100 at 23° and 30° respectively, which agree well with the values originally reported by Warburg & Christian (179) for rat muscle aldolase, but which are 2.5 times those reported by Taylor *et al.* (180). Baranowski & Niederland also studied the aldolase activity of a crystalline myogen-A preparation which was homogeneous in the electrophoresis apparatus and the ultracentrifuge. It was found to have from 15 to 35 per cent the activity of a standard aldolase preparation. It is thus clear that myogen-A cannot be considered a homogeneous protein and further it is not identical with aldolase.

Aconitase.—Aconitase of approximately 30 per cent purity has been prepared from pig heart muscle by Buchanan & Anfinsen (181). Low temperature alcohol and ammonium sulfate fractionation were employed in the presence of citric acid, which acted as a stabilizing agent. Saffran & Prado (182) found that transaconitate was an effective competitive inhibitor of aconitase. When added to tissue slices citric acid was found to accumulate.

Transaminases.—Pyridoxamine phosphate was found by Umbreit *et al.* (183) not to be active as coenzyme for purified apo-transaminase (and tyrosine decarboxylase). The possible rôle of the coenzyme as an intermediary amino group donor in the transamination reaction as originally suggested (184, 185) would thus seem to be uncertain. Muscle tissue from vitamin E deficient guinea pigs and rabbits has been found to have only one-half the transaminase activity of normal animals (186). The transaminase activity in germinating seeds and its relation to protein synthesis has been investigated by Smith & Williams (187). Evidence for the existence of a specific aspartic-pyruvic transaminase in pigeon and hen liver was reported by Kritzmann & Samarina (188).

Transmethylase.—Dubnoff & Borsook (189) have succeeded in partially purifying an enzyme from liver and kidney which catalyzes the anaerobic transfer of a methyl group from dimethylthetin or dimethyl-1-propiothetin to homocysteine. The enzyme is termed dimethylthetin transmethylase. This enzyme appears to be different from the betaine and choline transmethylase previously reported by these investigators (190) since it has a different pH stability. Steensholt (191) has reported that D-methionine is a more efficient methyl donor to ethanolamine and dimethylethanolamine with rat liver homogenates than is L-methionine.

Others.—Mention only can be made here of a few of a large number of reports dealing with the enzymatic cleavage of a variety of compounds. The enzyme causing the hydrolysis of triacetic acid to acetoacetic acid and acetic acid has been purified some 100-fold by Connors & Stotz (192). The enzymatic hydrolysis of triacetic acid lactone to triacetic acid has been demonstrated by Meister (193). The enzymatic hydrolysis of *p*-nitrophenol ethers (194) and phenol sulfates (195) has been reported. The anaerobic synthesis of alanine from histidine plus pyruvic acid by liver extracts takes place independently of transamination, according to Wiss (196). Reports from the same laboratory indicate that rat liver extracts can cleave kynurenine anaerobically to form anthranilic acid and alanine (197). The enzyme system responsible has been partially purified and shown to be specific for L-kynurenine (198). Rat liver extracts have been shown to contain an enzyme which catalyzes the breakdown of halogenated compounds, such as methylene chloride, to form formaldehyde, hydrogen and chloride ions (199). The enzyme has been partially purified and requires activation by cyanide or sulfhydryl compounds. The enzyme system effecting the conversion of ribose-5-phosphate to hexose-monophosphate has been partially purified by Waldvogel & Schlenk (200). The phosphoroclastic decomposition of acetoacetate to acetylphosphate and acetate has been studied with enzyme preparations of *Clostridium Kluyveri* by Stadtman & Barker (201).

The enzyme hydantoinase has been partially purified and its properties studied (202).

The successful use of a filter paper chromatopile for the separation of amylase, adenosine deaminase, and adenylic acid phosphatase from takadiastase has been reported by Mitchell *et al.* (203). Paper chromatography of proteins and enzymes was studied by Franklin & Quastel (204). Thorn (205) and Whittaker & Adams (206) have presented methods for determining the ratio of the Michaelis constants of enzymes with respect to two substrates.

Synthesizing Enzyme Systems

The anaerobic enzymatic synthesis in the presence of ATP of a number of compounds has been reported. While the enzyme systems involved are undoubtedly complex, they have been simplified to the extent that they are not dependent upon oxidative reactions. The synthesis of citric acid from acetate plus oxaloacetate in presence of ATP, coenzyme A, and Mg^{++} was

achieved by Stern & Ochoa (207) using an ammonium sulfate fraction of acetone-dried pigeon liver extracts. The synthesis of acetylcholine and a related compound by the choline acetylation system from rabbit brain has been studied by Nachmanson *et al.* (208). In addition to coenzyme A and ATP, this system requires cysteine (for aerobic incubation), Mg^{++}, and Ca^{++}. A colorimetric method for the estimation of acetylcholine using hydroxylamine was devised by Hestrin (209) and employed in these studies. Speck (210) has carried out a detailed study of the mechanism of glutamine synthesis using a purified pigeon liver preparation free of adenosinetriphosphatase. A stoichiometric relationship was established between the ammonia utilized, the glutamine formed, and the inorganic phosphorus liberated. Attempts to demonstrate the formation of an intermediate of the type of γ-glutamyl phosphate, however, were unsuccessful. Bloch & Johnson (211) have succeeded in demonstrating the anaerobic synthesis of glutathione from glutamic acid, cysteine, and glycine in the presence of ATP using extracts of acetone-dried pigeon liver. The glycine was labelled with C^{14} and synthesis was determined by measuring the radioactivity of the isolated glutathione. The enzyme system responsible for the synthesis of arginine from citrulline plus aspartic acid has been partially purified and shown to consist of two enzymatic steps by Ratner & Pappas (212). The first step involves the condensation of the substrates in the presence of ATP and Mg^{++} to form an intermediate which is hydrolyzed by the second enzyme to form arginine plus malic acid. The solubilization and partial purification of the enzyme which catalyzes the synthesis of citrulline from ornithine plus carbamylglutamic acid in the presence of ATP has been effected by Grisolia & Cohen (213). Glutamic acid, carbon dioxide, and ammonia cannot replace carbamyl glutamic acid in this system.

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CARBOHYDRATE CHEMISTRY[1]

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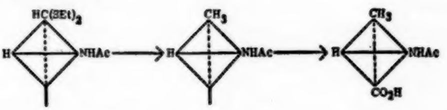
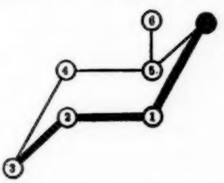
General: Sugars

Rotatory power and configuration.—An automatic saccharimeter has been constructed and used by the Spreckels Sugar Co. [Bernhardt, (1)]; 400 readings to ±0.01 per cent sucrose can be made and recorded per hour. Rotatory dispersions in the visible region on *p*-phenylazophenyl *β*-D-glycosides indicated that the isolated azo chromophore had no rotatory effect [Bonner, (2)]. Statistical evaluations show that "the molecular rotation of a 1,5-anhydroglycitol lies well between the molecular rotations of the anomeric methyl glycopyranosides of the corresponding aldose" [Fletcher & Hudson, (3)]. A comprehensive study of the rotations of the aldopentonamides and aldohexonamides at various concentrations and wave lengths established the Vant Hoff principle of superposition as a fair approximation [(Hockett *et al.* (4)] (Cf. 273).

By combining the crystal structure [Beevers & Stern, (5)] of (*dextro*)-tartaric acid with the observable face development of the crystal, a determination of absolute configuration is claimed [Waser (6)]. The result is the opposite of that presently selected but it is extremely doubtful if such a capricious property as habit can be employed in a valid manner for this purpose. Employing derivatives of D-glucosamine, L-(*levo*)-glyceraldehyde has been correlated with (*dextro*)-alanine by a direct chemical method [Wolfrom, Lemieux & Olin (7)].



Conductometric and polarimetric measurements on cuprammonium complexes of the pyranosides of D-glucose and D-galactose are compatible with the chair conformation of the ring [Reeves & Jung (8); Reeves (9)].



[1] This review attempts to cover the period essentially from October, 1948 to November, 1949, insofar as the journals were available to the authors.

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Occurrences and isolations.—D-Tagatose [Hirst, Hough & Jones (10)] and L-sorbose [Martin & Reuter (11)] are reported as hydrolytic products of the gum from *Sterculia setigera* and of the pectin in *Passiflora edulis*, respectively. L-Arabinose occurs free in the heartwood of red cedar, *Thuja plicata* [Anderson & Erdtman (12)]. D-Fructose is found in semen and a study has been made [Mann *et al.* (13)] of the amounts found in the bull at various stages of sexual development. Botanical classification based on isolable substances indicates that pinitol (methyl-*d*-inositol) is characteristic of all legumes; quebrachitol (methyl-*l*-inositol) of the *Sapindacea, Hippocastanacea,* and *Aceracea;* and dulcitol of *Celastracea* [Plouvier (14)]. Paper chromatography on the hydrolysates of sea urchin egg jelly demonstrates that L-fucose with or without D-galactose and D-glucose, parallels zoological classification [Vasseur & Immers (15)].

New glycosides reported are the D-glucoside paulownioside from the twigs of *Paulownia imperialis* [Chollet (16)]; a D-galactoside of luteolin from the flowers of *Chaerophyllum sylvestre* [Clemo & Felton (17)]; citrifoliol, a flavanone bioside of D-glucose and L-rhamnose from the fruits of *Citrus trifoliata* [Sannié & Sosa (18)]; an anthocyanin from the Mexican flower "macpalxochitl" that yielded on hydrolysis three moles of D-glucose and a like amount of gallic acid [Pallares & Garza (19)]; and meratin, a 3-di-D-glucosylquercetin from the flowers of *Meratia praecox* [Hayashi & Ôuchi (20)]. Isoquercitrin has been found in the seed pods of *Cercis canadensis* [Douglass *et al.* (21)]; isorhamnetin in the anthers of *Lilium candidum* [Tappi & Karrer (22)]; and pelargonidin 3-D-glucoside in strawberries [Sondheimer & Kertesz (23)].

A glucomannan containing a small amount of uronic acid constitutes the mucilaginous layer of the leaves of *Aloe vera* [Roboz & Haagen-Smit (24)]; another glucomannan is found in *Iles mannane* [Wise (25)]. Spherocrystals of fructans different from inulin are reported from *Arctium lappa* and *Veronica spuria* [Murakami (26)]. A new polysaccharide from black spruce (*Picea mariana*) contains D-galactose, L-arabinose and uronic acid [Brauns (27)]. Two new polysaccharides are found in *Penicillium luteum;* one is predominantly a galactan and the other a glucan (28, 29).

Rabbit hair contains significant amounts of glycogen (30). Contrary to Fraenkel & Jellinek (31), the chitin of *Limulus polyphemus* is almost certainly identical with that of other arthropods (32). It is suggested that chondroitinsulfate, acting as a multivalent anion, cements together the protein collagen molecules into fibrous macromolecules in nasal cartilage [Partridge (33)]. An assay for heparin in blood indicates a normal human blood content of 0.1 μg. per ml. [Jaques *et al.* (34)].

Sugars of cardiac glycosides.—Found in nature for the first time are 6-desoxy-D-allose, in gefruside [Keller & Reichstein (35)], and 6-desoxy-L-talose, in sarmentoside A [Schmutz (36)]. D-Diginose is identified as 3-methyl-2,6-didesoxy-D-*arabo*-hexose by synthesis from D-galactal [Tamm & Reichstein (37)]; its further occurrence in two glycosides is reported

[Rangaswami & Reichstein (38)]. Thevetose is shown to be 3-methyl-6-desoxy-L-glucose by synthesis from L-glucose [Blindenbacher & Reichstein (39)], thus establishing another noteworthy occurrence of the L-glucose structure in nature. Thevetose also occurs as a monoacetate [Frèrejacque & Durgeat (40)]. The synthesis of 2-desoxy-thevetose (oleandrose, 3-methyl-2,6-didesoxy-L-*arabo*-hexose) has been accomplished [Blindenbacher & Reichstein (41)]. An alternative synthesis of digitalose (3-methyl-6-desoxy-D-galactose) from methyl α-D-galactopyranoside is recorded [Tamm (42)]. The synthesis of 3-desoxy-D-idose (3-desoxy-D-*lyxo*-hexose) and 3-desoxy-D-gulose (3-desoxy-D-*xylo*-hexose) completes the series of 3-desoxyhexoses [Huber & Reichstein (43)]. D-Glucose can be cleaved enzymatically from cardiac glycosides (44, 45, 46); the enzyme is not identical with α- or β-D-glucosidase.

Phosphate esters.—General phosphorylation procedures have been critically examined [Atherton, Howard & Todd (47)]. An improved preparation of D-glucose 4-phosphate is recorded [Reithel & Claycomb (48)]. Phosphorylenolpyruvic acid (PEPA) has been synthesized from β-chloro-α-hydroxypropionic acid [Baer & Fischer (49)]. Monophosphates of inositol (common form) and scyllitol are obtainable [Iselin (50)] through reduction and phosphorylation of pentaacetyl-5,5-dideshydroinositol (*scyllo-meso*-inosose). A striking result has been the isolation, in impure form, of D-glucose-1,6-diphosphate, believed to be concerned with the conversion of α-D-glucopyranose 1-phosphate to D-glucose 6-phosphate (51, 52). The synthesis of the anomeric forms of this substance has been accomplished [Posternak (53)]. Experiments with O^{18}-enriched water demonstrate that the phosphate linkage in α-D-glucopyranose-1-phosphate cleaves between the carbon and oxygen on hydrolysis by acid and by either muscle or sucrose phosphorylase but cleaves between the phosphorus and oxygen with phosphatase [Cohn (54)]. Acetyl dihydrogen phosphate exhibits the first type of cleavage on alkaline hydrolysis and the second on enzymatic hydrolysis [Bentley (55)].

Cyclitols (inositols).—Müller's "*iso*-inositol" (56) is *dl*-inositol [Posternak (57); Fletcher & Findlay (58)]. The Wieland & Wishart (59) synthesis of inositol by the catalytic reduction of hexahydroxybenzene has been reinvestigated [Anderson & Wallis (60)]; inositol, scyllitol and a new cyclitol are reported. Efforts to replace the nitrogen functions with hydroxyl in the nitrodesoxyinositols obtained [Grosheintz & Fischer (61)] by the cycloaldolization of 6-nitrodesoxyaldohexoses have been unsuccessful [Iselin & Fischer (62)] but the aminodesoxyinositol obtained is of interest.

Synthesis of isotopically-labeled sugars.—Radioactive carbon dioxide is incorporable by photosynthesis into starch (tobacco leaf) or sucrose (*Canna indica* leaf). The radioactive α-D-glucopyranose 1-phosphate and D-fructose obtainable from these by enzymatic or acid breakdown are then synthesizable with sucrose phosphorylase into sucrose labeled in either or both monosaccharide components (63). 1-C^{14}-D-glucose can be prepared chemically by the Fischer-Kiliani method from C^{14}-(hydrogen cyanide) [Koshland &

Westheimer (64)] or from C^{14}-nitromethane by the Sowden-Fischer synthesis [Sowden (65)].

Photosynthesis.—Labeled components of cell extracts of the green algae *Chlorella* and *Scenedesmus* irradiated in the presence of $C^{14}O_2$, were separated by paper chromatography and located by radioautography on x-ray film. The first carbon dioxide-fixation products (5 sec.) are phosphoglyceric and phosphopyruvic acids. Free sucrose appears prior to any other unsubstituted sugar but is preceded by triose phosphates, α-D-glucopyranose 1-phosphate and D-fructose-6-phosphate. The usual glycolytic intermediates are thus involved [Calvin *et al.* (66 to 69)]. Labeled carbon in the plant monosaccharide after a short period of photosynthesis in $C^{14}O_2$ was predominantly on C_1 and C_6; with longer periods the distribution over all carbons was about equal [Gibbs (70)].

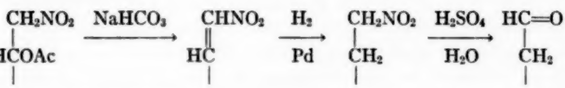
General analytical.—Infrared spectroscopy offers great promise in carbohydrate analysis [Trotter *et al.* (71); Kuhn (72); Burket & Badger (73)]. Polarography may be useful for D-fructose assay [Vavruch (74)]. The Kiliani reaction has been adapted to reducing sugar determination through Kjeldahl assay of nitrogen fixed as ammonia [Frampton & Smith (75)]. The rate of reaction with cyanide is a function of sugar structure and pH [Militzer (76)]. An exact alkaline copper oxidation procedure has been devised and applied to a study of the variation of oxidizability with sugar configuration [Heidt *et al.* (77)]. New apparatus and techniques for the semimicro determination of uronic acids are described [Maher (78)]. *cis*-Glycols are potentiometrically titratable with lead tetraacetate [Reeves (79)]. A color reaction for glycolaldehyde [Dische *et al.*, (80)] and new specific color reactions [Dische *et al.* (81)] for hexoses are reported. The Dische reaction for 2-desoxypentoses is shown to depend upon the formation of ω-hydroxylevulinaldehyde (82). The anthrone-sulfuric acid reagent is useful for sugar assay (83, 84) and the molybdenum blue reaction is useful for the analysis of reducing sugars in the presence of sugars that are nonreducing (85, 86). Fructose is colorimetrically determined in the presence of glucose by its greater capacity to reduce the phosphotungstate-phosphomolybdate reagent of Folin-Denis (87). Spectrophotometric studies are recorded for sugars in sulfuric acid alone (88) and in the presence of orcinol, carbazole or skatole (89, 90). Procedures are given for the analysis of inulin in plasma and urine (91) and for starch (92) and pectic substances (93) in plants; cellulose may be determined by acid dichromate oxidation (94). Methods are published for the detection and determination of lactose in urine and food products (95 to 98). An improved mucic acid assay for galactose is claimed to give 90 per cent yields (99). D-Xylose and L-arabinose can be determined together by selective fermentation (100).

Synthesis of Sugars and Sugar Derivatives

Synthetic methods.—D,L-Arabitol and ribitol are formed in a series of steps from 2-penten-4-yn-1-ol [Raphael (101)] and D,L-threitol from crotonaldehyde diacetate [Schmid & Grob (102)]. Several crystalline glycoside deriva-

tives of D-glucuronic acid are obtainable by the oxidation of methyl α-D-glucopyranoside with dinitrogen tetroxide [Hardegger & Spitz (103)]. *meso*-Tartaric acid is formed from N_2O_4-oxycellulose (celluronic acid) by successive periodate and chlorate oxidation with subsequent hydrolysis [Head (104)]. Oxidation of D-glucose to D-glucosaccharic acid is effected in aqueous solution with air and a platinum catalyst [Mehltretter, Rist & Alexander (105)]. At 165° C. and 5,000 p.s.i. the Adkins copper chromite catalyst reduces dialkyl tartrates to an isomerized mixture of tetritols, albeit diethyl L-(*dextro*)-tartrate does give a 65 per cent yield of crystalline L-threitol and dimethyl *meso*-tartrate produces crystalline erythritol in 70 per cent yield [Trenner & Bacher (106)]. The pure tetritols alone are isomerized under these conditions. It is annoying that the authors employ the term "erythritol" for "tetritol."

The glycal method for the synthesis of the D and L forms of D-*erythro*-2-desoxypentose is reinvestigated and improved (107). Iodide replacement of methanesulfonates on secondary alcohol groups would lead to intermediates of value in desoxy sugar synthesis. Although this reaction is reported for C_4 in D-glucose [Helferich & Gnüchtel (108)] it fails for D-arabinose [Overend & Stacey (109)]. The 2,3-anhydropentoses are useful for the synthesis of 2- or 3-desoxypentoses (110, 111, 112). D-*erythro*-2-Desoxypentose is obtained [Sowden (113); Overend, Stacey & Wiggins (114)] by application of procedures established in the 2-desoxyhexose series [Sowden & Fischer (115)]. The principle of this synthesis is that the acetate of a 1-*C*-nitroalcohol loses acetic acid readily to form a 1-nitro-1-olefin which on hydrogenation, deacetylation and nitrogen cleavage, with cold sulfuric acid, yields the desired compound.



The acetylated alkali-sensitive glycosides of 3-phenyl-4-hydroxycoumarin are easily made from the silver enolate of the aglycon and an acetylglycosyl halide. They undergo catalytic transglycosidation with Walden inversion and offer possibilities for the synthesis of glycosides of the α configuration [Spero, Ballou & Link (116)]. Boron trifluoride in chloroform at room temperature converts the acetylated alkyl β-glycosides to the α-anomers [Lindberg (117)]. A very remarkable reaction is the conversion [Lindberg (118)] of a β-1,6-disaccharide linkage to the α-1,6 form with titanium tetrachloride in chloroform [Pacsu (119)]; in this manner gentiobiose was transformed to isomaltose. Acetylated β-D-glycosides of phenols can be made from β-D-glucose pentaacetate and boron trifluoride in anisole solution [Bretschneider & Beran (120)]. Substitutes were sought for silver compounds as condensing agents in the Koenigs-Knorr reaction; mercuric cyanide was very effective [Helferich & Wedemeyer (121); Lindberg (122)]. Although the first product

of the reaction of D-galactose and D-mannose with ethanethiol is the thioacetal, longer reaction times yield anomeric mixtures of the thiopyranosides [Fried & Walz (123)].

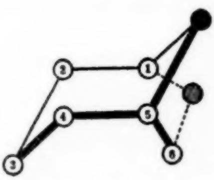
An interesting type of sugar derivative is the acetylglycosyl azide obtained by reaction, with apparent Walden inversion, of the acetylglycosyl halide with silver azide; reduction yields the 1-amino derivative [Bertho (124)].

Trifluoroacetic anhydride is reported as a powerful impelling agent for esterification with carboxylic acids; it is claimed that starch and cellulose can be fully esterified in 60 min. at 50°C. [Stacey *et al.* (125)]. Olefin formation may be avoided in the replacement of tosyloxy groups by iodine through the use of acetic anhydride as a solvent (126, 127). Contiguous tosyloxy groups, one of which is primary and the other secondary, are both removed on treatment with sodium iodide in acetone with the production of a double bond [Hann, Ness & Hudson (128)]. This reaction has been exploited in derivatives of D-mannitol to yield D-*arabo*-3,4,5,6-tetrahydroxyhexane and D-*threo*-3,4-dihydroxyhexane [Karrer & Davis (129)]. Hypohalite addition to the olefin linkage led to the synthesis of sorbitol from D-mannitol and of L-iditol from sorbitol [Bladon & Owen (129a)].

General preparation and synthesis.—Studies on the kinetics of sucrose crystallization are continued [Whelan, Galkowski & Van Hook (130)]. Crystalline D,L-glucose is reported for the first time [Wolfrom & Wood (131)]. D-Tagatose is prepared by the improved alkaline isomerization of D-galactose and in a high state of purity by the oxidation of D-talitol with *Acetobacter suboxydans* [Totton & Lardy (132)]. Perseulose (L-galaheptulose) is synthesized from L-galactonic acid [Wolfrom, Berkebile & Thompson (133)] and two new ketooctoses from the epimeric D-galaheptonic acids [Wolfrom & Cooper (134)]. Bertrand's "volemulose" (135) is identified as D-altroheptulose (sedoheptulose) and it is shown that whereas D-altro-D-*manno*-heptitol (volemitol) is oxidized by *A. suboxydans* to a mixture of D-mannoheptulose and D-altroheptulose [Ettel & Liebster (136)], only the latter is produced when the heptitol is oxidized by *A. xylinum* [Stewart, Richtmyer & Hudson (137)]. The supposed 3-ketohexose "glutose" has no real existence (138). Carob gum serves as a good source of D-mannose [Smith (139)]. Trehalose is preparable from bakers' yeast [Elander & Myrbäck (140)] or waste brewers' yeast [Stewart, Richtmyer & Hudson (141)]. Sources of tetroses have been studied (113, 114). [See also (107 to 115).]

Oxo-sugars.—Calcium melibionate and 2-ketolactobionate are prepared [Walton & Isbell (142)]. Oxidation of D-glucosone produced no formaldehyde; some type of hemiacetal structure involving C_5 or C_6 must then be present [Becker & May (143)]. [See also (103 to 105).] An improved preparation of crystalline reductone (glycerosone 2,3-enediol) from D-glucose by the action of sodium hydrogen plumbite is reported and further studies of this reactive substance are made. Similar treatment of dihydroxyacetone is stated to lead to the formation of its enediol (reductol), characterized as a crystalline bis (*p*-nitrobenzoate) [v. Euler *et al.* (144, 145)].

Anhydro sugars and anhydro glycitols.—A crystalline dianhydro sugar obtained as a byproduct from a wood-gas plant is perhaps 1,4:3,6-dianhydro-D-glucopyranose; on hydrolysis it yields 3,6-anhydro-D-glucose [Tishchenko & Nosova (146)]. New derivatives of 3,6-anhydro-D-glucose are reported (147). Epoxy formation can be effected by alkaline treatment of a sulfate ester with Walden inversion on the ester carbon; 1,6-anhydro-β-D-galactopyranose 2-sulfate is thus converted to 1,6:2,3-dianhydro-β-D-talopyranose [Duff (148)]. Attention is directed to the fact that a *trans* configuration on C_2 and C_3 (altrose and idose) leads to spontaneous 1,6-anhydro ring formation [Wiggins (149)]. Cuprammonium complex formation studies allow the assignment of a chair ring conformation to 1,6-anhydro-β-D-aldohexopyranosides [Reeves (150)]. [See also (35 to 43, 110 to 112).]



Reductive desulfurization of thiopyranosides leads to the synthesis of the 1,5-anhydro derivatives of lactitol, maltitol, ribitol [Fletcher, Hudson, *et al.* (151, 152)] and D-mannitol (styracitol) (123). Acid treatment of D-ribamine yields a crystalline anhydro-D-ribitol of probable 1,4 structure [Kuhn & Wendt (153)]. A number of dianhydrohexitols and their derivatives are described (154 to 159).

Cyclic acetals.—Derivatives of sorbitol containing ethylidene groups in the 4,6 (160), 2,4, 1,3:2,4 and 1,3:2,4:5,6 (161) positions are described. 1,2:5,6-Dicyclohexylidene-D-glucofuranose has been reported (162). Another case of benzylidene migration is established (163).

Allyl ethers.—Allyl ethers of sucrose and D-mannitol have been further described and polymerized [Yanovsky *et al.* (164, 165)].

Acyclic esters.—Mild acetylation of D-psicose (D-ribohexulose) yields the *keto*-acetate [Binkley & Wolfrom (166)]. Carbonyl reduction of an *aldehydo*-acetate is difficult but has been effected for *aldehydo*-D-ribose tetraacetate [Fox (167)]. *Aldehydo*-D-xylose hexaacetate has been obtained from the acetolysis of xylan and *aldehydo*-D-galactose heptaacetate from guaran [Whistler, Heyne & Bachrach (168)]. [See also (133, 134).]

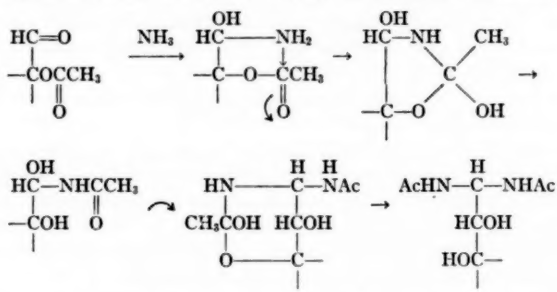
Glycosides.—The natural disaccharide glycosides fabiatrin, primulaverin, gein and vicianin have been synthesized [Robertson *et al.* (169, 170, 171)]; the sugar of the first two is primeverose, 6-(β-D-xylopyranosyl)-D-glucose, and that of the latter two is vicianose, 6-(α-L-arabopyranosyl)-D-glucose. [See also (35 to 46), (116 to 123)].

Nitrogen derivatives.—Reductone (glycerosone 2,3-enediol) forms stable compounds with aniline and *l*-leucine [v. Euler *et al.* (172)]. Further aryl carbamate esters of sugar derivatives are reported [Wolff & Rist (173)].

Carbohydrates chemically bound to proteins have been synthesized in several ways [Micheel *et al.* (174 to 177)]. The carboxyl group of cellulose glycolic acid ether was converted to the azide which coupled, in mild alkali, with gelatin and the pseudoglobulin of horse serum; soluble products containing *ca.* 20 per cent carbohydrate (orcinol assay) and insoluble products containing *ca.* 90 per cent carbohydrate were obtained. In a variation of this procedure, the azide was reacted with tyrosine ethyl ester and the product, as azide, was coupled with the same proteins. Similar procedures were effected by operating on the carboxyl groups of cellobiose glycolic acid glycoside, lactobionic acid, and gum arabic; products containing 5 to 35 per cent carbohydrate were obtained. Smaller amounts of carbohydrate (1 to 5 per cent) were introduced by reacting albumins with D-glucosylcyanamide (prepared in solution from the ureide); the product formed was probably the D-glucosylguanidino-protein.

Miscellaneous

General reactivity.—The conception of "neighboring groups" reactivity was established by Fischer (178) to explain acyl migration. It has of late received considerable elaboration. Diacetamido derivatives are intermediates in the Wohl degradation. Their formation from *aldehydo*-acetates and ammonia is explained [Isbell & Frush (179)] by the intermediates shown below.

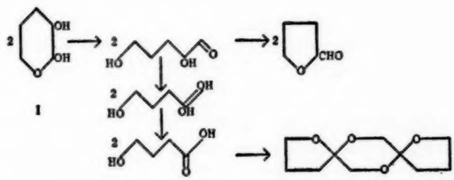


Similar conceptions are applied further to the formation of glycosides and orthoesters from acetylglycosyl halides; a solvated orthoester intermediate is postulated in the glycosidation of tetraacetyl-α-D-mannosyl bromide (a "*trans*" halide) and under conditions of lower solvation the acetylated α-D-glycoside may be obtained in 34 per cent yield [Isbell & Frush (180)]. The known acetylglycosyl halides of the hexoses are stable only in the α anomer and undergo replacement by alkoxyl with Walden inversion. However, tribenzoyl-β-D-ribopyranosyl bromide, $[\alpha]_D$ -199° (chloroform), is a stable substance and its bromine atom undergoes facile alkoxyl exchange under slightly acidic conditions [Jeanloz, Fletcher & Hudson (151, 181)]; the 1,2-orthobenzoate is a probable intermediate and a double Walden inversion occurs with apparent retention of configuration. The known stable triacetylarabinosyl halides are likewise β-isomers that undergo halogen-alkoxyl exchange with Walden inversion and formation of the α-glycosides. The rather

obscure reaction by which phenyl tetraacetyl-β-D-thiopyranoside is converted to α-D-glucopyranose pentaacetate by bromine in acetic acid is studied [Bonner (182)].

As previously cited (116), certain enolic glycosides undergo alkaline methanolysis to produce methyl glycosides with Walden inversion; other alkali-sensitive enolic glycosides, as that of theobromine, undergo cleavage under like conditions to yield the methoxy-aglycon [Ballou & Link (183)]. Another alkali-sensitive glycoside is that of β-hydroxypropiononitrile [Helferich & Weber (184)]. The chemical production of lactic acid by alkaline cleavage of sugars is reviewed [Montgomery (185)]. Sodium in toluene cleaves two moles of methanol from dimethyl tetramethyl-D-glucosaccharate to form dimethyl 2,5-dimethoxymuconate [Wiggins (186)].

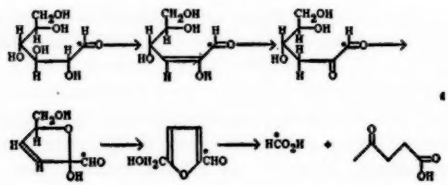
Pyrolysis of I, a sugar analogue, leads in part to the following reactions [Hurd & Edwards (187)].



It is claimed that D-mannitol forms two acidic complexes with boric acid but the exact evaluation of the stoichiometry and equilibria concerned is still an open question (188, 189). The reaction of boric acid with polysaccharides offers interesting possibilities (190, 191).

The aldonic and uronic acids are successive intermediates in the oxidation of galactose to mucic acid [Pigman *et al.* (99)]. D-Glucono-δ-lactone is not necessarily an intermediate in the hypoiodite oxidation of D-glucose in alkaline solution [Ingles & Israel (192)]. No 2,3-osazone is detectable in the reaction of D-fructose with phenylhydrazine [Binkley & Wolfrom (193)]. Evidence is presented which suggests that R-CHOH-O-CO_2H is a probable intermediate in the Ruff degradation [Karrer & Haab (194)].

Browning reaction.—The interaction of amino acids or proteins with reducing sugars to produce brown colorations and, finally, black pigments, is of considerable significance and is known as the "browning," melanoidin or Maillard (195) reaction (196 to 200); a preliminary fluorescence (201) is involved. Dried fruits (202), in particular, apricots [Stadtman *et al.* (203, 204, 205)], (206, 207), skimmilk (208), roasted peanuts (209), and cane molasses (210), have all been studied from this standpoint. Sugar decomposition products are believed to be concerned in this reaction. Breakdown into smaller carbon fragments (210) and into furan bodies (211) have been considered as reaction sequences. The acid breakdown of reducing sugars to furans has been studied spectroscopically (211) and, in part, by isotopically marked sugars (212). Evidence is established for the following reaction sequence involving the facile α,β-dehydration of carbonyl compounds.



Sugar separations.—The separation of sugars or of their partially methylated derivatives, especially in polysaccharide hydrolysates, continues to be a major problem. Alcoholysis of methylated disaccharides with acid benzyl alcohol and subsequent separation and hydrogenolysis of the benzyl glycosides, serves as a method for obtaining the monosaccharide ethers [Coleman *et al.* (213)]. Tetra-, tri- and di-methyl ethers of D-fructose are separable on the silica-water column with toluene-ethanol [Bell & Palmer (214)]. The operative ease of "paper chromatograms" and their adaptation to small quantities of material, makes them presently very popular; ammoniacal silver nitrate, benzidine, aniline acid phthalate and alkaline permanganate have been used as spot indicators (215 to 220). For isolation or quantitative ends, this technique is not so suitable, albeit some attempts at microanalysis of the spot material have been made (221, 222, 223); [see also (66 to 69)]. Column chromatographic procedures with carbon, silicates, silica or alumina as adsorbents are more useful for larger scale separations (224).

Methylation reference compounds.—Methyl 3,5-ditrityl-D-arabofuranoside offers an alternative route to 2-methyl-D-arabinose [Halliburton & McIlroy (225)]. Methyl D-ribofuranoside from guanosine served as a source for 2,3,5-trimethyl-D-ribose anilide, 3,5-dimethyl-D-ribophenylosazone, and the lactone and phenylhydrazide of 2,3,5-trimethyl-D-ribonic acid [Barker (226)]. 3,5-Isopropylidene-D-xylose diethyl thioacetal can be employed for the preparation of 2,4-dimethyl-D-xylose [Dalley & McIlroy (227)]. 4-Methyl-D-xylophenylosazone is described [Wintersteiner & Klingsberg (228)].

POLYSACCHARIDES

Homopolysaccharides.[3]—Floridean starch occurs in many red algae; it consists entirely of D-glucose and periodate assay indicates that the linkage is 1,3. It is resistant to β-amylase [Barry *et al.* (229)]. A water-soluble dextran formed from *Betacoccus arabinosaceous* possessed a highly branched structure since on methylation and hydrolysis it yielded 2,3,4,6-tetramethyl-D-glucose (1 part), 2,3,4-trimethyl-D-glucose (3 parts) and 2,3-dimethyl-D-glucose (1 part) [Stacey & Swift (230)]. The preparation of dextrans from *Leuconostoc mesenteroides* is detailed; the products are rather heterogeneous [Jeanes *et al.* (231, 232)]. Partially hydrolyzed dextran is designated as a blood plasma substitute [Ingelman & Halling (233)]. A levan produced from sucrose by *Pseudomonas mors-prunorum* produced on methylation and methanolysis

[3] Other than cellulose, starch, and glycogen.

about 9 per cent methyl 1,3,4,6-tetramethylfructofuranoside and methyl 1,3,4-trimethylfructofuranoside (identified by paper-strip chromatography); this indicates a chain of about twelve fructofuranose units mutually joined through positions 2 and 6 [Gilbert & Stacey (234)]. The extensive work of Schlubach on plant fructans is continued; partial structures for the products from wheat straw and barley spikes are given (235, 236). Crystalline xylan (barley straw and birchwood) and mannan A (ivory nuts and slash pine) preparations are claimed [Yundt (237)]. Crystalline hexaacetylxylobiose was isolated by chromatographic techniques [Bachrach & Whistler (238)]. [See also (168) and (258)].

Heteropolysaccharides.—Ultracentrifuge sedimentation velocity runs showed one polysaccharide component in the heartwood water extract of western larch and the same component (probably) and another in the sapwood extract [Borgin (239)]. The hydrolysis products of methylated cholla gum and egg plum gum have been identified. The aldobiuronic acid 6-(D-glucuronosyl)-D-galactose is obtained from gum arabic, egg plum gum and almond-tree gum [Brown, Hirst & Jones (240)]; that of the seed mucilage of *Plantago ovata* Forsk is 2-D-galacturonosyl-L-rhamnose [Laidlaw & Percival (241)]. Partial structures for the seed mucilage of *Plantago lanceolata* [Percival & Willox (242)] and the hemicellulose of flax [McIlroy (243)] are recorded. The galactomannan (guaran) (244, 245) of guar seed can be enzymically hydrolyzed (246). Methylation data are incomplete but indicate that essentially all of the D-galactose is in terminal units (247), in harmony with periodate data (248). [See also (168).] The galactomannan of carob gum contains D-mannose (80 per cent) and D-galactose (20 per cent); methylation and hydrolysis gives 2,3,4,6-tetramethyl-D-galactose, 2,3,6-trimethyl-D-galactose and 2,3-dimethyl-D-galactose [Hirst & Jones (249); Smith (250)]. It is therefore a chain of D-mannose units to which is attached an occasional terminal D-galactose entity. Two galactomannans may be present in locust bean gum [Smith *et al.* (251)].

The structure of chondroitinsulfuric acid (from cartilage) was attacked by periodate assay on the initial substance, the partially acid-desulfated product, and the hydrolysate of the methylated polysaccharide; a 1,3 linkage results [Meyer *et al.* (252)]. The inhomogeneity of heparin preparations is further demonstrated by counter-current techniques (253).

Pectin.—The methyl glycoside of pectin methyl polygalacturonate probably has a chain length of about 30 D-galacturonic acid residues (254). Gelation properties are hindered by acetylation (255, 256); beet pectin contains an acetyl group (255a, 257). The araban accompanying sugar beet pectin is like that of the peanut; the L-arabinose units are present one-third as end groups, one-third linked 1,5 and one-third linked 1,3 [Hirst & Jones (258)]. A small amount of L-rhamnose, D-galactose and L-arabinose may be an integral part of the pectin molecule (256).

Starch[4] *and glycogen.*—The amylose-amylopectin separation in starches

[4] A considerable amount of significant work has been omitted as not of apparent biological relevance.

may be effected in an amazing variety of ways (259); the former is readily determined by its characteristic iodine absorption (260). Sago starch is a good source of amylose (261); waxy maize starch has less than 2 per cent of amylose (262). Sweet corn (golden bantam variety) contains a starch very similar to animal glycogen (263). The molecular sizes of derivatized starch fractions is determined by osmotic methods (264, 265, 266); natural glycogens from various animal sources show high molecular sizes as estimated by light scattering methods (267). The streaming birefringence of amylose solutions is interpreted as due to elongation of coiled molecules (268). Evaluation of the nonreducing terminal groups in amylopectin and glycogen by tetramethyl-D-glucopyranose assay (269) and by determination of periodate-produced formic acid (270, 271, 272) continues with good agreement between the two methods. Aryl carbamates of starch, glycogen, and dextrins have been prepared; they show a difference in rotation which appears to be a function of the degree of branching [Wolff & Rist (273)]. Highly chlorinated products are prepared from starch and liquid chlorine [Barham & Thomson 274)].

Crystalline α- and β-amylases are now available from a variety of sources (275, 276). The existence of a third enzyme, "limit-dextrinase," capable of breaking down the limit dextrins, is indicated (277 to 281). Maltase-free pancreatic amylase attacks randomly both amylose and amylopectin, the latter being more open to attack in the early stages; maltose is immediately detectable and D-glucose is detectable in the later stages (282, 283). Evidence is presented that β-amylase produces maltose and maltotriose but no D-glucose (284).

Considerable indirect evidence exists for C_6 as the point of branching in amylopectin and glycogen but it is desirable to place this on a more definitive basis through degradative experiments. The dextran from a suitable strain of *L. mesenteroides* is known to be a linear polymer containing α-1,6 linkages. The disaccharide unit, 6-(α-D-glucopyranosyl)-D-glucose or isomaltose, was obtained from this source, in the form of its crystalline β-D-octaacetate, by chromatographic methods applied to the acid hydrolysate; the structure of the disaccharide was verified by periodate oxidation of its glycoside and several crystalline derivatives were prepared [Wolfrom, Georges & Miller (285); see also (118)]. The same substance is obtained on processing the acid hydrolysate of glycogen (rabbit liver) [Wolfrom & O'Neill (286)]. Action of α- and β-amylases of malt on amylopectin (waxy maize starch) does not yield isomaltose but maltotriose has been isolated as a crystalline β-D-hendeca-acetate and its structure established by methylation and enzymic techniques [Wolfrom *et al.* (287, 288)]. When amylopectin was hydrolyzed by an enzyme preparation (Takamine type) from the mold *Aspergillus oryzae,* isomaltose was isolable in crystalline form [Montgomery, Weakley & Hilbert (289)]; levoglucosan was also produced (290).

The α- and β- and γ-Schardinger cyclic dextrins have been prepared by improved procedures and cleaved with acids to yield various products

[Myrbäck *et al.* (291, 292)], including a sirupy maltohexaose [French *et al.* (293, 294)]. The action of *Bacillus macerans* amylase is apparently reversible (295, 296).

The phosphorylases of animal (rabbit muscle) and plant (potato) sources synthesize products similar to amylose from α-D-glucopyranose 1-phosphate. Other enzymes or co-enzymes are required to synthesize the branched structures, such as glycogen or amylopectin. Enzyme systems are known which convert sucrose [Hehre *et al.* (297)] or maltose [Torriani & Monod (298)], respectively, into polysaccharides of the starch-glycogen class without the intermediate formation of α-D-glucopyranose 1-phosphate.

Cellulose.[4]—Pear cell-wall cellulose is chemically identical with cotton cellulose [Hirst *et al.* (299)]. The electron microscope shows that cellulose fibers built by *Acetobacter xylinum* are similar to cell-wall plant cellulose and have a thickness of 250 to 400 Å [Mühlethaler (300)]. Complex molecular size distribution curves were obtained on fractionation of nitrated bamboo cellulose (301). Cellulose-decomposing bacteria have been isolated from deteriorated cotton fabrics [Siu *et al.* (302)] and from the intestinal contents of a porcupine (303).

Cellulose has considerable orientation as evidenced by Debye-Scherrer x-ray diffraction patterns; various preparations of this substance possess such orientation in varying degree and are considered to be composed of ordered ("crystalline") and unordered ("amorphous") regions. Various methods of estimating the degree of "crystallinity" are based upon x-ray data [Hermans & Weidinger (304); Tsien (305); Heyn (306)] and upon chemical procedures dependent upon the assumption that the less-ordered regions react heterogeneously more readily [Svedberg (307); Meller (308); Timell (309)]; they are complicated at times by phenomena believed to indicate "recrystallization" tendencies [Hermans *et al.* (310, 311)].

The question of the bond homogeneity of cellulose and whether or not one bond out of about 500 is more susceptible to reagent attack, as in acid hydrolysis, continues to be debated *pro* and *con* [(Husemann & Goecke (312); Mehta & Pacsu (313); Reeves *et al.* (314, 315)]. The existence of carboxyl groups in unmodified cellulose is now denied [van der Wyk & Studer (316); Ant-Wuorinen (317)]. A polymer-homologous series of crystalline α-D-acetates from a degree of polymerization of one through six has been isolated from cellulose acetolysates by chromatographic methods [Dickey & Wolfrom (318)]. Aqueous hydrochloric and sulfuric acids are effective for cellulose hydrolysis but sulfurous and phosphoric acids are not [Harris & Kline (319)].

In cellulose derivatization, 2-methylcellulose (degraded but fibrous) is reported [Sugihara & Wolfrom (320)]; disulfide cross links are introduced into cellulose esters [Izard & Morgan (321)]; cellulose xanthate thioanhydrides and polymerized allylcellulose are reported [Danilov *et al.* (322, 323)]. The relative reactivity of the cellulose hydroxyls toward various reagents are studied [Timell (324)]; primary hydroxyl content in partially substituted

derivatives may be assayed by its greater reactivity in some reactions [Tasker & Purves (325); Malm, Tanghe & Laird (326, 327); Hayes & Lin (328)]; C_2 and C_6 are always more reactive than C_3 though their reactivity may depend upon reagent and conditions.

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CHEMISTRY OF LIPIDS[1,2]

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The subject of lipid chemistry continues to be a fascinating one. New fatty acids have been isolated from various natural sources and studies on the distribution of fatty acids in the triglyceride molecule have been expanded. An attractive theory to account for the arrangement of the fatty acids in neutral fat has recently been proposed. Further information is available on the inositol-containing phospholipid which can be prepared from both vegetable and animal sources. The importance of fats from a nutritional standpoint, as well as their rôle in protein metabolism, has recently been emphasized (1, 2, 3). Fats therefore should be considered as an obligatory constituent rather than as an optional component of the diet.

In addition to the treatise of Markley (4) which appeared in 1947, new monographs include a book on fatty acids and their derivatives by Ralston (5) which is especially valuable for information on products closely related to fatty acids. In addition to the new edition of *The Chemical Constitution of Natural Fats* by Hilditch (6), noteworthy works are Warth's *Chemistry and Technology of Waxes* (7) and the excellent opus of Karrer & Jucker (8) on *Carotinoide*. Less comprehensive reviews include one by Hilditch (9), an annual review by Piskur (10), and two by Daubert (11, 12).

FATTY ACIDS AND TRIGLYCERIDES

Fatty Acid Distribution in Triglycerides

There has been much discussion in the recent years regarding the rules which govern the distribution of the fatty acids in the triglyceride molecules. Previous to this year three possible theories for explaining the arrangement of such fatty acids had some measure of support. Doerschuk & Daubert (13) have now proposed a theory which enables one to calculate the composition of the triglyceride fractions in corn oil with considerable precision.

Mono-acid theory.—The so-called mono-acid theory was first suggested as an explanation of the composition of natural fats. According to this idea, fats are made up of simple triglycerides. Some time ago this hypothesis was largely abandoned as a satisfactory explanation of fat composition because of the inability of many workers to isolate more than traces of such simple triglycerides, even though, on the basis of fatty acid composition, a large proportion might be expected. Moreover, the demonstration of the

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widespread distribution of mixed triglycerides (containing two or three different fatty acid residues) in various natural fats has proved that types of glycerides other than simple ones occur in nature.

Theory of "even distribution."—The most widely accepted theory is that of Hilditch (6), which has been called the "rule of even distribution." The author describes the pattern of "even distribution" as follows:

If an acid, *A*, is present to the extent of 33 per cent or less of the total fatty acids, it will occur not more than once in the triglyceride molecules. If, on the other hand, it forms 33 to 67 per cent of the total fatty acids, two molecules may be present in some of the triglyceride molecules. The frequency with which it is present to the extent of two molecules is increased as the concentration of *A* approaches 67 per cent. Finally, it is only when this acid, *A*, exceeds 67 per cent that it will make up an appreciable proportion of the simple triglyceride. As the amount of acid, *A*, is increased above this value, the proportion of it present as the simple triglyceride obviously becomes greater. Although this rule of even distribution is not adhered to with mathematical rigidity, Hilditch points out that this generalization fits a vast majority of natural fats. However, this worker (14) reported earlier that small proportions of simple triglycerides do occur in seed fats where the total concentration of the acid, *A*, is less than 67 per cent. Moreover, in previous work (15, 16) it was noted, also, that acid, *A* might be present in the triglycerides to the extent of two molecules in the case of cottonseed oil and in the oils of certain citrus seed fruits, when only about 25 per cent of the total acids were composed of the acid, *A*. However, allowing for these slight discrepancies, the principle of even distribution has been found applicable to most land vegetable fats, to the fats from aquatic fauna and flora, which include the larger fishes and marine mammals, and to the fats of many land animals.

Theory of random distribution.—A third theory to explain the triglyceride constitution is that of random arrangement. According to this idea, the fatty acids combine with glycerol in an order based upon the laws of chance, and the distribution in these cases can be readily calculated from mathematical equations. Although it is obvious that this chance distribution cannot be applied to most vegetable fats, there are two classes of animal fats which would seem to follow such a pattern, namely, milk and animal depot fats which are rich in stearic acid. Hilditch and his co-workers (17, 18, 19) originally suggested that saturated acids are distributed in the triglyceride molecules of such fats in a random arrangement, and it was first believed that radically different principles govern the synthesis of such animal fats from those which are operative in vegetable fats. As a result of more recent studies, Hilditch and his co-workers have now concluded that the glyceride structure of the above fats may be consistent with the superimposition of certain chemical changes on the original mixture of palmitic and oleic acid which was assembled in the form of mixed triglycerides according to the usual principles of even distribution. It is suggested (6, 20 to 24) that in the animal depot fats which are rich in stearic acid, the oleoglycerides are in part

converted into stearoglycerides by a process described as biohydrogenation. Similarly, the original oleoglycerides of milk fats are converted into the short-chain saturated and unsaturated glycerides characteristic of such fats (6, 25 to 32).

On the other hand, Longenecker (33) is of the opinion that the distribution of the fatty acids in the animal fats occurs on the basis of the law of probability. He has indicated that the synthesis of vegetable and of animal fats may be based on entirely separate mechanisms. This latter view has recently been supported by Norris & Mattil (34) who suggested that the enzymes responsible for the esterification of the fatty acids with glycerol may operate quite differently in animal and in plant systems. As a consequence, the variations in composition between the vegetable fats and the animal fats (milk fats and body depot fats containing a large proportion of stearic acid) can be explained. However, Hilditch (6) pointed out that even though a random distribution might be accepted as the pattern for the saturated fatty acids in these particular animal fats, there is no experimental evidence to indicate that a similar chance distribution obtains with the unsaturated fatty acids either in the vegetable or in the animal kingdom. Moreover, this author finds that (35) in the constituent glycerides of a number of ox, sheep, and pig body fats, there is no correlation in the proportions of di- and tri-unsaturated glycerides with a random distribution, although the ratios of the tri-saturated and the mono-unsaturated groups of glycerides do agree fairly well with those calculated from probability. According to this author there is no need to postulate different enzyme syntheses of the glycerides in the vegetable and animal cells.

Hilditch agrees that the proportions of the four types of mixed triglycerides (GS_3, GS_2U, GSU_2, GU_3, where S represents a saturated acid, and U represents an unsaturated acid) coincide with those calculated on probability considerations, in a number of different instances. This approximation to a random distribution may actually be accidental rather than an indication that such an arrangement is the governing factor. For example, when the proportion of saturated or of unsaturated acids in the fat is comparatively high, the values calculated for the tri-saturated glycerides, either on the basis of even distribution or on a random basis, become almost identical. Experimental errors in the determinations are sufficient to make it impossible to state which of the two patterns is being followed. This is the case with the tri-unsaturated glycerides of pig-back fat (22) as well as with the tri-unsaturated glycerides of the Indian sheep (36) and Indian cow fats (21). Furthermore, it is impossible to determine from experimental values whether even distribution or a random pattern obtains in the case of seed fats having 80 to 85 per cent of saturated acids (6) as well as of the nut fats of the *Palmaceae*, such as babassu fat (37). A second example in which a pattern of fatty acid distribution based upon the even principle may be confused with one based upon the random arrangement occurs when the total number of fatty acids exceeds four. A clear-cut pattern of even distribution is most readily observed with those fats in which the major fatty acid components

are limited to three or four. In the case of fats in which six or seven different fatty acids occur in about the same proportions, calculation of the distribution will be approximately similar, whether it is based upon the even distribution principle or upon a random arrangement. Hilditch & Maddison (38) have recently demonstrated that in certain marine animal oils in which fatty acids such as palmitic, hexadecenoic, oleic, eicosenoic, eicosatetraenoic, eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids may be present, the mixed triglycerides are assembled as would be expected from the operation of even distribution. On the other hand the proportion of the tri-saturated, mono-, di-, and tri-unsaturated glycerides likewise agrees fairly closely with a random distribution. Hence, Hilditch believes that many fats in nature which are essentially evenly distributed may appear to have a random distribution.

Finally, Hilditch has discounted the application of his own experiments [Bhattacharya & Hilditch (19)] in the controversy. In these earlier tests it was demonstrated that the synthetic tri-glycerides formed by heating glycerol at 145°C. in a vacuum in the presence of traces of an aromatic sulfonic acid with various mixtures of acids such as lauric, palmitic, stearic, and oleic, were assembled as mixed triglycerides having a random distribution. The same would apply to the earlier results of Norris & Mattil (39) which were concerned with the distribution of the fatty acids following interesterification. The latter authors had noted that when soybean oil, cottonseed oil, a mixture of tripalmitin and triolein, or one of lard and hydrogenated lard, were heated with a small proportion of a stannous hydroxide at 225°C. for a short period, a rearrangement occurred so that the newly-formed triglycerides approximated the pattern indicative of chance distribution. These experiments confirmed the results of the direct esterification tests of Bhattacharya & Hilditch (19). Similar data have also been reported by Naudet & Desnuelle (40) with substrates of either oleodistearin and stearodiolein, or of tristerain and triolein. The products obtained when equimolecular mixtures of tristearin and triolein were so treated contained 11.3 per cent of tristearin, 38.6 per cent oleodistearin, 39.2 per cent stearodiolein, and 10.9 per cent triolein, compared with values based upon random distribution of 12.5 per cent each for the tristearin and triolein and 37.5 per cent each for the two mixed oleostearins.

Hilditch (9) concludes that the results obtained by esterification of glycerol with mixtures of fatty acids in the test-tube or from interesterification of mixtures of selected triglycerides do not contribute any information as to the behavior of enzymes in the living cell. Obviously the chemical reactions involved require high temperatures and other conditions incompatible with enzyme activity. Thus, if one accepts the validity of such criticisms, one is forced to the conclusion that the process of even distribution may be a universal one and that the variations from this rule in certain animal fats may be reconciled by the hypothesis that such fats are formed from a biohydrogenation of oleopalmitins previously laid down in conformity with

the law of even distribution. On the other hand, if one accepts a chemical viewpoint as the guiding principle in explaining physiological processes, one may still interpret the divergencies in composition of vegetable and animal fats as due to variations in the lipolytic enzymes of plants and animals.

Theory of partial random distribution.—Doerschuk & Daubert (13) have suggested still another pattern for the assemblage of the triglycerides, which would seem to be somewhat of a compromise between random and even distribution. These authors refer to the new scheme as a "partial random pattern." Corn oil was separated into a series of 19 different fractions by the use of low temperature solvent precipitation from acetone. The composition of each fraction was determined from the proportion of unsaponifiable residue, iodine value, saponification equivalent, melting point, and the refractive index. The amounts of dienoic and trienoic acids were determined spectrophotometrically by ultraviolet absorption, while the proportions of monenoic acid (oleic) and of saturated acids were determined by difference. The efficacy of their experimental procedures is attested to by the fact that they were able to recover 982 gm. of the original total of 1 kg. of corn oil used. There were four main fatty acids present in the corn oil. From their original kilo., 979.6 gm. of glycerides were accounted for, which consisted of 140.8 gm. of saturated acids, 219.6 gm. of oleic acid, 569.4 gm. of linoleic acid, and 5.88 gm. of linolenic acid, giving a total of 935.7 gm. of total acid.

On the basis of random distribution, 40 chemically different triglycerides can be formed from the above four acids, as calculated from the expression $(n^3+n^2)/2$ in which "n" represents the number of different fatty acids. However, since it is impossible to distinguish between positionally different glyceride isomers, the total number of constitutionally different triglycerides is preferably employed. The number of such derivatives may be calculated from the expression $(n^3+3n^2+2n)/6$.

Only six possible mixed triglycerides may occur if the even distribution pattern is followed. These are *Lo, Lo, S* (2); *Lo, Lo, Ol* (2); *Lo, Lo, Ln* (2); *Ln, Lo, Ol* (3); *Ln, Lo, S* (3); *S, Ol, Lo* (3) (the figure in parentheses represents the number of positional isomers, and the acids are as follows: *Lo*, linoleic acid; *Ln*, linolenic acid; *Ol*, oleic acid; and *S*, saturated acids). The number of mixed triglycerides is limited to this number because of the following considerations. Since oleic, linolenic and the saturated acids are each present in less than a 33 per cent concentration, only one molecule of each can occur in a triglyceride. In the case of linoleic acid, which is present in an amount between 33 and 67 per cent, one or two molecules may be present in each triglyceride.

In the partial random distribution pattern, Doerschuk & Daubert (13) have postulated not only that some triglyceride molecules may occur in which linoleic acid is absent, but also that oleic, linolenic and the saturated acids may occur twice in some triglyceride molecules. Under such conditions, 16 mixed triglycerides might occur. The proportions of each of the 20 constitutionally possible glycerides actually determined in corn oil are

compared, in Table I, with the proportions of each which might be expected if mono-acid distribution, the principle of even distribution, a random arrangement, or a partial random distribution were operative.

TABLE I

A COMPARISON OF THE TRIGLYCERIDES FOUND IN CORN OIL WITH THOSE CALCULATED ON THE BASIS OF DIFFERENT THEORIES ON TRIGLYCERIDE PATTERNS OF FAT*

Triglyceride structure†	Number of positional isomers	Triglycerides from 1 kg. corn oil calculated and found				
		Mono-acid triglyceride	Random distribution	Even distribution	Partial random pattern	Experimentally determined
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
Ln, Ln, Ln	1	6.15	0.003	0	0	0
Lo, Lo, Lo	1	596.00	223.00	0	8.00	8.90
Ol, Ol, Ol	1	230.00	12.50	0	0	0
S, S, S	1	147.00	3.24	0	0	0
Ln, Ln, S	2	0	0.02	0	0	0
Ln, Ln, Ol	2	0	0.03	0	0	0
Ln, Ln, Lo	2	0	0.07	0	0	0
Lo, Lo, S	2	0	164.00	291.00	332.00	335.00
Lo, Lo, Ol	2	0	257.00	524.00	485.00	482.00
Lo, Lo, Ln	2	0	6.96	0	0.50	0
Ol, Ol, S	2	0	23.90	0	1.69	3.60
Ol, Ol, Lo	2	0	98.00	0	70.00	72.70
Ol, Ol, Ln	2	0	1.02	0	0.50	0
S, S, Ol	2	0	15.30	0	4.16	0.18
S, S, Lo	2	0	39.90	0	17.50	16.80
S, S, Ln	2	0	0.41	0	3.20	4.10
S, Ol, Lo	3	0	125.00	146.00	40.80	41.50
Ol, Lo, Ln	3	0	5.34	15.50	1.00	0
S, Ol, Ln	3	0	1.30	0	10.50	11.80
S, Lo, Ln	3	0	3.40	3.00	2.94	3.19

* Adapted from Doerschuk & Daubert (13).

† Ln = linolenic acid radical; Lo = linoleic acid radical; Ol = oleic acid radical; S = saturated acid radical.

The proportion of mixed triglycerides actually found in corn oil agrees well with the partial random theory and very poorly with the conception of even distribution or random arrangement. The results indicate that mono-acid formation cannot be a valid theory. It will be interesting when this technique is applied to other vegetable fats and to animal fats. This compromise proposal seems to offer a scheme which may afford a better explanation for the method of assemblage of the fatty acids in both vegetable and animal fats than any of the current theories.

Composition of Various Vegetable Fats

In addition to the comprehensive investigation of Doerschuk & Daubert (13) on corn oil, a number of studies of other fats have been carried out. Kapur & Daubert (41) made an extensive investigation of a number of oils belonging to the *Cruciferae*. These included the oil from yellow mustardseed (*Brassica alba*), black mustardseed (*B. nigra*), and rapeseed (*B. campestris*). In addition to determining the proportions of the various acids, the authors found that a C_{20} mono-ethenoic acid (probably 11-eicosenoic) hitherto not reported was present in the black mustardseed oil. A hexadecenoic acid was found in one fraction of rapeseed oil. Baliga & Hilditch (42) reported the presence of a number of unsaturated acids in rapeseed oil which included 11-eicosenoic acid ($CH_3(CH_2)_7CH:CH(CH_2)_9COOH$), and 13,16-docosadienoic acid ($CH_3(CH_2)_4CH:CHCH_2CH:CH(CH_2)_{11}COOH$), and 9-hexadecenoic acid ($CH_3(CH_2)_5CH:CH(CH_2)_7COOH$). The first occurred to the extent of 5 per cent, and the two latter approximately 1 per cent. Small amounts of a polyethnoid C_{16} acid with traces of lower unsaturated acids, probably containing 14 carbon atoms, were also found.

Clopton and co-workers (43) reported myristic acid and possibly palmitic acid in okraseed oil, as well as 7.9 per cent of arachidic acid, and a possible isomer of linoleic acid. Swift (44) has carried out an analysis of the oil from the seeds of the tangerine (*Citrus nobilis var. deliciosa*). In general, the composition of the oil compared quite closely with that of the oils obtained from the seeds of the grapefruit and the orange, but the tangerine seed oil contained more linolenic acid.

The seed fat of the Bacury plant (*Platonia insignis* mart.) has been found to differ in composition from most seed fats in the high content of palmitic acid (55 per cent) (45). Only two other seed fats of the *Guttiferae* family, namely *Madhuca butyracea* and *Caryocar villosum*, have previously been shown to be rich in palmitic acid. Appreciable amounts of palmitic and stearic acids have also been noted in the oil from the seeds of *Juffa aegyptiaca*, which is a tropical *Curcurbita* (46). Baliga & Meara (47) have reinvestigated the component fatty acids and glycerides of dhupa fat (so-called Malabar tallow) obtained from *Vateria indica*. The analyses agree well with the earlier ones (48, 49). This composition corresponds closely to that of other members of the same botanical family (*Dipterocarpaceae*). Mono-oleo-disaturated glycerides formed 64 to 78 per cent of the component glycerides, and 40 to 46 per cent of these consisted of oleodistearin. Di-oleo-mono-saturated glycerides made up 16 to 34 per cent of the total, and 13 to 25 per cent of these consisted of stearodiolein.

Munguia and collaborators in Mexico City have reported the composition of several seed fats from trees common to that region. These include the seed oils from the Caoba or mahogany tree (*Swietenia macrophylla Kina*) (50), Habilla de San Ignacio (*Hura polyandra Baill*), a tree native to Sinaloa (51), and the Napahuite tree (*Trichilia Hirta L.*) (52) which has seeds resembling peanuts. These oils all contain saturated glycerides making up 25 to 32 per cent of the total oil. In the Napahuite, linoleic acid and oleic acid

glycerides are present in about the same proportions, 32 and 34 per cent respectively. In Caoba oil the content of linoleic acid glycerides is twice that of oleic acid glycerides (48 versus 24 per cent), while in Habilla oil linoleic acid glycerides are three times as abundant as those of oleic acid (52 versus 16 per cent). The oil of the Mamey (*Calocarpum mammosum L.* Pierre), which is a well-known Mexican fruit, has a composition quite similar to that of the seed oils (53).

Another unusual fat which has recently been analyzed (54) is the kernel fat of the lalob fruit (*Balanites aegyptiaca*). Linoleic acid was present in the largest amount (43.8 per cent as triglyceride), while oleic (30.5 per cent) and saturated acids (23.7 per cent) made up the other chief acids. This fat carries a considerable amount of α-carotene. Wheat germ from the Argentine (55) has a composition similar to that of samples obtained from other sources recorded in Hilditch (6). Murti & Dollear (56) have reported fatty acid composition from the bran of the Arkansas-grown Zenith variety of rice as follows: linolenic acid 0.84 per cent, linoleic 33.1 per cent, oleic acid 46.3 per cent, saturated acids 17.1 per cent, and unsaponifiable matter 2.7 per cent. The oils obtained from two varieties of filberts (Barcelona and Du Chilly) raised on the Pacific coast have a composition which agrees quite well with that of the European varieties. However, Fang & Bullis (57) found arachidic acid for the first time in this oil, as well as a C_{20} to C_{22} monoethenoid acid which has not been further identified. Linolenic and oleic acids have each been shown to make up about a third of the triglyceride fraction of dehydrated alfalfa leaf meal, (58) with linoleic making up 17 per cent, and the saturated acids about 20 per cent.

Milner (59) has determined the fatty acids present in an alga (*Chlorella pyrenoidosa*). Polymyxin A, from *Bacillus polymyxa*, has been shown to contain an optically active fatty acid with a probable empirical formula $C_9H_{18}O_2$ (60). Gore & Petersen (61) also found this acid, which differs from pelargonic acid in containing more methyl groups. Weitzel (62) has suggested that the branch-chain acids such as tuberculostearic, phthioic, and phytomonic acid may be metabolic products which work as metabolic antagonists.

Composition of Animal Fats

Hilditch & Pathak (63) have made a comprehensive study of the composition of the blubber and liver lipids of the common seal (*Phoca vitulina L.*). The composition of the blubber oil varied considerably from that obtained earlier (64) in the gray seal (*Holichoerus grypus*) in having a lower percentage of C_{14} and especially C_{16} unsaturated acids, and a markedly higher quantity of C_{22} unsaturated acids, as well as in having a small amount of C_{24} unsaturated acid.

Fresh body fat of an Indian sheep has recently been investigated by Hilditch & Shrivastava (36), using the more recent methods of Riemenschneider *et al.* (65) for the segregation of mixed triglycerides by crystallization from appropriate solvents. The fully saturated triglycerides (28 per

cent) consist chiefly of dipalmitostearins (16 per cent) and palmitodistearins (11 per cent). Very small proportlons of tripalmitin and tristearin may be present. In the mono-saturated glycerides (28.6 per cent) the chief component is oleopalmitostearin, while small proportions of oleodipalmitin and perhaps hexadecenopalmitostearin may also occur. The di-unsaturated glycerides make up the largest component (40.5 per cent), of which about 26 per cent consists in palmito-di-unsaturated and 14 per cent stearo-di-unsaturated glycerides. Only 3 per cent of the total mixture is made up of tri-unsaturated glycerides. Indian sheep body fat seems to differ from that of the English sheep body fat in containing somewhat more trisaturated glycerides and definitely more di-unsaturated glyceride groups, with a correspondingly smaller proportion of mono-unsaturated glycerides.

Sell and collaborators (66) reported the presence of myristic, palmitic and stearic acids and of the unsaturated acids, palmitoleic, oleic, and linoleic acids in the depot fat of the North American black bear. With the exception of stearic acid, these acids were identified as the chief components of the lipids from the scent gland of the Louisiana muskrat (67). In addition, C_{24} and C_{26} unsaturated acids were also present to the extent of 8.5 per cent, while decanoic, dodecanoic, stearic, tetracosanic, dodecenoic, and tetradecenoic acids were present to less than 1 per cent. Reference has already been made to the constituent glycerides of ox, sheep, and pig body fats by Hilditch (35), in relation to the prevailing pattern of triglyceride structures.

New Fatty Acids and Related Compounds

The presence of 11-eicosenoic acid has been reported in amounts of 5 per cent in rapeseed oil (42), and has been noted as a component of the liver oil from the Atlantic cod (*Gadus callarius*) (68). This is of especial interest since earlier workers have found only the 9-eicosenoic acid in liver oil of the Pacific cod. Hopkins *et al.* (68) have also reported the 9-eicosenoic acid in Jojoba oil, mustardseed oil, hog fat, and a number of seed fats. Methyl arachidonate has been isolated by chromatographic adsorption from the fatty acids from the lipids of the adrenal gland (69). Hawke (70) prepared ricinoleic acid in 99 per cent purity by crystallization from acetone at $-70°$C. from hydrolyzed castor oil fatty acids. Candelilla wax has been found to contain straight-chain acids, chiefly C_{28}, C_{30}, C_{32}, and C_{34} (71). The C_{32} acid made up more than 20 per cent of the fraction. Lemon (72) prepared isolinoleic acid from hydrogenated linseed oil by crystallization at low temperatures followed by chromatography using silica gel. *l*-Ascorbylpalmitate has been shown to have an $[\alpha]_D^{25.5°}$ of 23.3°, while *l*-ascorbyllaurate gave a specific rotation at 25.5° C. of 26.6° (73).

The isolation of α-monopalmitin from hog pancreas has recently been described by Jones *et al.* (74). They consider that this monoglyceride is a normal constituent of living pancreatic tissue. Although it was found in the amount of 1.9 per cent in the pancreas, practically none could be demonstrated in brain, adrenal, or liver tissues.

Synthesis of Fatty Acids and Fats

In addition to the method for the synthesis of fatty acids from petroleum products developed to a considerable extent during World War II (75), another method for the total synthesis of fatty acids and fatty aldehydes has been proposed by Grundmann (76), by condensation of crotonaldehyde under the influence of pyridine. On hydration of the resulting products and condensation with malonic acid, lauric acid and cetyl alcohol were produced, and in some cases, myristic and stearic acids. New methods for the synthesis of vaccenic acid (*cis* and *trans*-11-octadecenoic acid) (77), for the preparation of erucic acid, and of its geometrical isomer, brassidic acid (78), and for a series of oxy- and keto-fatty acids (79), have been reported during the year. The preparation, in a 78 per cent yield from castor oil, of 12-ketostearic acid is reported (80). Methyl ricinoleate is first reduced to 12-hydroxystearate, which is oxidized to the 12-ketostearate and saponified to obtain the free keto-acid. Cason and his co-workers have reported the synthesis of a number of different branched-chain fatty acids. These include the 14-ethylhexadecanoic acid, 15-ethylheptadecanoic acid (81), 20-ethyldocosanoic acid, 18-*n*-propylheneicosanoic acid, 12-*n*-hexyloctadecanoic acid, and 9-*n*-octylheptadecanoic acid (82). A series of acids with methyl side chains were likewise prepared. These included 2-methyloctadecanoic acid, 2-methyldocosanoic acid, 3-methyloctadecanoic acid, 3-methyltetracosanoic acid, 4-methyloctadecanoic acid, and 2,3-dimethyloctadecanoic acid (83). A series of monoglycerides and mono-esters of polyhydric alcohols has been synthesized by Savary (84). The mono-esters were obtained when eight hydroxyl groups were present for each carboxyl group. Fatty acid esters of glycol, glycerol, pentaerythritol, and dipentaerythritol, were prepared with soap as a catalyst. Wocasek & Koch (85) studied the catalytic effect of various fluorides in the condensation of glycerol and fatty acids. After 15 hr., glycerol was combined with 72, 83, and 84 per cent of stearic, oleic, and linoleic acids respectively, without a catalyst. The stearate was present largely as a distearate, while a larger proportion of oleate and about half of the linoleate occurred as triglycerides. Of the 15 metallic fluorides studied, only antimony trifluoride, and zinc and cobalt fluorides exhibited outstanding catalytic properties. When zinc fluoride was used as a catalyst, glycerol was combined with 95, 94, and 92 per cent of stearic, oleic, and linoleic acids respectively. The chief compounds produced were triglycerides.

Methods for the Analysis of Fatty Acids and Fats

Kretchmer (86) has reviewed methods for the analysis of fatty acids, fats, phospholipids, and sterols, and has suggested a microprocedure for carrying out all these analyses on small amounts of animal tissues. A rapid method for the determination of free and combined fatty acids and fat in feces has been described by van de Kamer *et al.* (87), while Bauer and associates (88) have described a colorimetric method for the determination of the total esterified fatty acids in serum.

Chromatographic and other adsorption methods for the determination of fatty acids.—Recent procedures for the separation of fatty acids by chromatographic analysis and by counter-current distribution have been excellently described by Lovern in the last Review (89). Since then, Moyle *et al.* (90) have described a satisfactory method for the separation, identification, and estimation of most of the steam-volatile members of the saturated series of the fatty acids, using the principle of partition chromatography. They have employed heavily buffered silica gel without an indicator as the stationary phase of the partition column, with a mixture of chloroform and butanol as the liquid phase. Although this procedure allows a quantitative separation of the fatty acids from acetic to caprylic acid, it does not permit the separation of caprylic from the higher fatty acids. Similarly the unnatural fatty acid, pelargonic (C_9), can be separated from the lower members of the series but not from the higher fatty acids. The earlier work of Ramsey & Patterson (91) dealt with separation and determination of the straight-chain saturated fatty acids from C_5 to C_{10} by the use of a silicic acid column with a methanol-isooctane mixture as the immobile solvent; this was later modified by Ramsey (92) for the separation of *n*-butyric and isobutyric acids. It has been extended to permit the separation of the saturated straight-chain fatty acids from C_{11} to C_{19} (93). This has been made possible by the use of a mixture of furfuryl alcohol and 2-aminopyridine as the immobile solvent and *n*-hexane as the mobile phase on the silica gel column. Riemenschneider and co-workers (94) separated pure methyl linoleate and methyl linolenate by fractional adsorption on silicic acid. Adsorption of the homologous series of straight-chain fatty acids on the resin, Duolite A-2, has been studied by Robinson & Mills (95). They found that acetic, propionic, *n*-valeric, and *n*-caproic acids are adsorbed from acetone-water solution in an increasing degree as the molecular weight becomes greater. The adsorption of soaps of lauric, myristic, palmitic, and stearic acids from aqueous solutions by carbon black increases with an extension of chain length in the case of the saturated acids (96). Sodium oleate is adsorbed somewhat more strongly than sodium myristate, but less readily than sodium palmitate. These authors have demonstrated that the adsorption of the soaps is a combination of two independent processes, namely (*a*) that of the neutral soap, and (*b*) of the fatty acid resulting from hydrolysis of the soap. Holman & Hagdahl (97) have employed a procedure designated as displacement analysis (98) for the concentration of a fatty acid from Stillingia oil. They had earlier made a fair separation of the C_{12} to C_{22} acids by displacing the fatty acids from a column of charcoal and Hyflo Super Cel with cetyl pyridinium chloride or picric acid.

Other methods for the estimation of fatty acids.—Koehler & Hill (99) have proposed a microdistillation procedure for determination of fatty acids, while Fawcett (100) has described the use of high vacuum distillation for the processing of oils. A microanalytical method has recently been described for the determination of volatile fatty acids (101), in which distillation is replaced by microdiffusion. This has the advantage in allowing simultaneous

determination of a large number of samples with a simple apparatus and with a minimum of manipulation. The individual acids cannot be determined separately, although a procedure is described for the separation of the hydroxy- and keto-acids from the fatty acids.

Harva & Ekwall (102) have suggested potentiometric titrations of the fatty acids with silver nitrate as a method for estimation of lauric, myristic, palmitic, stearic, and oleic acids. The unsaturated fatty acids of soybean oil can be concentrated when the fatty acids are neutralized with a mixture of 40 per cent sodium hydroxide and 60 per cent barium hydroxide (103). The resulting insoluble soaps contain largely saturated acids while the 35 per cent soluble soaps which are present in the filtrate contain the fatty acids with the high iodine number. In the case of the fish oils examined, the use of a mixture of 60 per cent sodium hydroxide and 40 per cent barium hydroxide resulted in a yield of about 50 per cent of the total fatty acids in the filtrate which had an iodine number of 283. Although this procedure is not satisfactory for the quantitative estimation of the different acids, it provides an excellent method for segregation of the saturated acids from those having a high iodine number. Singleton (104) described a technique whereby palmitic and oleic acids can be separated from each other when present in varying proportions in *n*-hexane solution or in acetone solution at low temperatures. The intersolubilizing effect of oleic acid on palmitic acid is greater in hexane than in acetone.

White & Brown (105) have suggested an interpolation method for the estimation of linolenic acid by the hexabromide method. It has long been recognized that the original procedure of Steele & Washburn (106) for the calculation of the amount of linolenic acid as 37.3 per cent of the weight of the bromides introduces very large errors because of the fact that, on bromination, linolenic acid yields ether-insoluble bromides which amount only to circa one-fourth to one-third of the theoretical yields. The hexabromide number consequently corresponds to 70–90 instead of to the theoretical 272. Shinowara & Brown (107) suggested that the linolenic acid percentage could be calculated by dividing the hexabromide number by 0.92; this figure was later modified to 0.96 (108). In the new method the linolenic acid content of fatty acid mixtures is obtained by interpolation from a curve in which the amount of linolenic acid brominated is plotted against the hexabromide precipitates. Since this curve takes into account the empirical nature of the hexabromide yield over the entire range of values, it should enable one to obtain accurate results with both low and high proportions of linolenic acid. A similar procedure has also been described by White, Orians & Brown for the estimation of arachidonic acid from the polybromide (109).

Physical and Chemical Properties of Fatty Acids and Fats

Polymorphism.—Malkin & Wilson (110) have recently reported on the polymorphic forms present in symmetrical mono-oleyl- and mono-elaidyl di-saturated triglycerides as determined by x-ray and thermal examination

of the products. These authors concluded that 2-oleodistearin, 2-oleodipalmitin and 2-oleodimyristin exist in five solid modifications, i.e., vitreous, α, β'', β' and β in order of their ascending melting points. They consider the reason for divergent results reported by others is probably due to the presence of diglycerides in the products. On the other hand, Jackson & Lutton (111) reported only three polymorphic forms each in a number of the saturated mixed glycerides (2-myristodipalmitin, 2-myristodistearin, 1-stearodimyristin, 1-palmitodimyristin). In the case of 2-stearodipalmitin, only two polymorphic forms were observed, while four polymorphic forms were reported for 2-myristodistearin. Jackson & Lutton (111) believe that only three of the four types postulated by Malkin are authentic. On the basis of a recent reexamination of the structure of a series of 1,3-diglycerides from dilaurin through distearin, Baur *et al.* (111a) have concluded that only two polymorphic forms of this type of diglyceride occur, which are designated, in conformity with Malkin's terminology, β-a and β-b. The first is obtained from the melt and sometimes from solvent crystallization. It is stable at room temperature but transforms to β-b when heated approximately to the melting point. A constancy in spacing as determined by x-ray examination was found, which does not conform with the earlier report of Malkin and co-workers (111b).

Interesterification.—Although it has been known for many years that when fats are heated at a high temperature for a long period a rearrangement of the fatty acids in the triglycerides takes place (112), it has more recently been recognized that the same change can be accomplished at much lower temperatures by the use of catalysts. Acids, bases, compounds of alkali and alkaline earth metals, water, and the metals tin, zinc, cadmium, and lead have been used for this purpose (113). Although sodium ethylate has been shown to be active at temperatures of 275 to 400°F. (114, 115), this is much higher than is necessary for interesterification, and is undesirable since other reactions are likewise catalyzed. Eckey (116) has shown that interesterification takes place rapidly at temperatures below 120°F. with sodium alkoxide as a catalyst in proportions of the catalyst as low as 0.1 per cent or less, provided that the glyceride mixture is dry, acid-free, and peroxide-free before the addition of the catalyst, and that the catalyst is dispersed rapidly throughout the mixture at the moment of addition. To allow a satisfactory ester interchange, air and moisture must be excluded from the reaction vessel. When interesterification takes place at a temperature sufficiently high so that none of the reaction products crystallize, the resultant fatty acid pattern of the triglycerides is one of complete random distribution. However, Eckey has shown that when interesterification is carried out at a lower temperature so that one of the reaction products can crystallize, it is possible to modify the composition of the resultant mixture markedly from that showing random distribution. Thus, when the reaction is carried out slightly below the temperature at which tristearin will crystallize, practically all the stearic acid in a fatty mixture is separated out in the form of tristearin. Under conditions so controlled, the procedure is designated a "directed

interesterification." Eckey & Formo (117) have recently employed directed interesterification for the production of monoglyceride. When cottonseed oil was used as the triglyceride mixture with sodium methoxide in xylene as the catalyst, and excess glycerol was added, 27 per cent of the fat was separated as the crystalline monoglyceride after three days at 26.7°C. On recrystallization from petroleum ether at 4.4°C., this was shown to be essentially pure. Farkas and his co-workers (118) studied the rate of trans-esterification of a simple system consisting of butyl alcohol and ethyl acetate as catalyzed by acids. When interesterification is produced by the use of absolute alcohol in the presence of sodium hydroxide (119), practically all of the fatty acids are changed to ethyl esters in 20 min., with an alcohol to fat ratio of two to one and 0.8 per cent of catalyst. Such an "alcoholysis" proceeds best at a temperature of 50°C. The alcoholysis reaction has also been used for producing the fatty acid esters of methyl glucoside (120).

Alkali conjugation of unsaturated fatty acids.—When unsaturated fatty acids are saponified for a prolonged period, a shift in the double bonds occurs and the unconjugated acids are partially converted to conjugated ones. Kass & Burr (121) were able to isolate pseudo-elaeostearic acid from saponified linseed oil after such treatment. Alkali is known to produce several types of conjugation. In the case of an acid with four double bonds, not only does tetraene conjugation result, but also triene and diene types. Holman & Burr (122) have recently found that at 178°C., the maximum triene, tetraene, and pentaene conjugation from the respective unconjugated acids occurs when potassium hydroxide is present in an amount of 22 to 23 gm. per 100 ml. The optimum conjugation reaction is completed in 8 min. Conjugation does not result from heating alone in the absence of alkali in the case of arachidonic and linolenic acids, but it does take place with equal facility in the fatty acid oxidation products subjected to heat or to alkali isomerization (123).

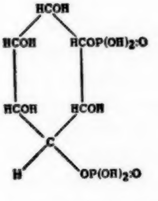
Hydrogenation of fats.—The hydrogenation of fats may follow different patterns with changes in the procedure. On the one hand, the addition of hydrogen may occur in a haphazard manner, or under controlled conditions, specific double bonds may be affected. The first type is called "nonselective," and the latter "selective," hydrogenation. Selective hydrogenation is favored when a catalyst is present and when agitation of the fat occurs during the addition of the hydrogen. Hilditch & Stainsby (124) demonstrated that one unsaturated linkage of linoleic acid is hydrogenated before any of the oleic acid is attacked. By altering the conditions of hydrogenation, it is possible to cause the production of oleic acid from linoleic acid, or "iso-oleic" acid, by initial saturation of the 9–10 double bond of linoleic acid. Hilditch (125) has shown that the hydrogenation of di- and polyunsaturated glycerides is selective. In the case of methyl elaeostearate, two molecules of hydrogen were added simultaneously with the production of 11-octadecenoic acid (126). No stearate was produced from methyl elaeostearate until 80 per cent of this product had been converted to the monoethenoid acid. Bailey (127) has worked out equations to determine the pro-

portion of various acids which can be hydrogenated under specific conditions. In the case of cottonseed oil, the ratio of the rate of hydrogenation of linoleic acid to that of oleic acid varies from 4:1 in the nonselective type to about 50:1 in selective hydrogenation. The rates of reaction of several acids in nonselective hydrogenation are as follows: oleic acid, 1; isolinoleic acid (9,15,-octadecadienoic acid), 2.5; linoleic acid, 7.5; and linolenic acid, 12.5. On the other hand, the rates for selective hydrogenation were found to be 1, 3.85, 31, and 77 respectively for the above acids. Soybean oil behaves similarly to linseed oil as regards the selectivity of hydrogenation. As an example of selective hydrogenation, Swern & Scanlan have produced an oleic acid of high quality by crystallization and distillation of fatty acids obtained from selectively hydrogenated animal fats (128, 129).

CONJUGATE LIPIDS

New Compounds

Although the fractionation of brain cephalin was actually predicted in the review article of Folch-Pi & Sperry (130), a final description of the products has only recently appeared (131, 132). In earlier work it was proved that brain cephalin is a mixture of several phosphorus-containing lipids (133) which consisted of phosphatidylethanolamine and phosphatidylserine, subsequently shown to be oleylstearylglycerylphosphorylserine (134). The third fraction of brain cephalin has now been shown to be a mixture which includes an inositol-containing phosphatide named diphosphoinositide (131). Folch (132) concludes that it is a meta-compound with the following structure:



This phosphatide accounts for all of the inositol present in brain cephalin. It appears to be a combination of inositol, metadiphosphate, glycerol and fatty acids in equimolecular proportions. It is not believed that any nitrogen occurs in the pure compound. The inositol metadiphosphate isolated by a short-time hydrolysis of the diphosphoinositide was determined from its elementary composition, by titration with alkali, by the isolation of inositol from it, and by the study of the products of reaction with periodic acid. On the basis of the latter reaction, the meta position was established.

By the use of counter-current distribution apparatus, Scholfield and collaborators (135) made a further study of "soybean lecithin," and concluded that the proportion of the several components in one sample of soybean

phosphatides was lecithin, 29 per cent; cephalin, 31 per cent; and inositol phosphatides, 40 per cent. They believed that two different types of inositol phosphatides were present. Whether one of these is identical with the diphosphoinositide described by Folch is uncertain. Folch & Arsove (136) demonstrated a new lipid in brain, which has an unidentified primary amine group and which becomes water-soluble after a short acid hydrolysis. It apparently contains sphingosine, galactose, a primary amine, and small amounts of fatty acids. A number of new synthetic choline derivatives have been described by Kubiczek & Neugebauer (137). These include fluorocholine, phenacetylcholine, and naphthacetylcholine as bromide and chloride derivatives.

Occurrence of conjugated lipids in tissues.—The distribution of the essential lipids in the gray and white matter of the brain of cats, dogs, beavers, and in humans has been examined by Johnson, McNabb & Rossiter (138). The results in the different species were quite uniform in demonstrating a greater concentration of cerebroside, total cholesterol, and total phospholipid in the white matter than in the gray matter. It was found that, when calculated on the basis of total essential lipid, the concentration of cerebroside and of total cholesterol was greater in white matter than in gray matter, but the concentration of total phospholipid was lower. However, of the total phospholipid fraction, the concentration of sphingomyelin was greater in the white matter. This was offset by a decrease in concentration of both lecithin and cephalin. The higher proportion of cholesterol in the white matter is in harmony with the findings of a number of investigators, while the demonstration of a greater proportion of cerebroside and sphingomyelin in white matter as compared with gray matter was reported earlier (139, 140). The lipids of the normal brain of the guinea pig, rat, and rabbit were shown to consist of circa 2 per cent cerebrosides (2.29, 1.88, 2.74), circa 2 per cent cholesterol (1.76, 1.94, 2.21), and circa 5 per cent phospholipids (4.82, 4.57, 5.40). "Cephalin" made up about one-half of the total phospholipids, while one-fourth each consisted of lecithin and sphingomyelin (138). In a later communication, Johnson and co-workers (141) compared the concentration of the various lipids in the brain of the infant and of the adult human subject. A considerably higher concentration of cerebrosides, free cholesterol, and sphingomyelin was found in the lipids of white matter of adult as compared with infant brain. Since these lipids are known to be present in high concentrations in the peripheral medullated nerves, it seems probable that cerebroside, free cholesterol, and sphingomyelin, rather than lecithin and cephalin, are the principal lipid components of the myelin sheath of the nerve fiber. Study of *in vitro* degeneration of calf brain slices showed that there was a considerable decrease in total phospholipid but no change in the concentration of cerebrosides or of total cholesterol. In the case of the phospholipids, sphingomyelin and cephalin were decreased in concentration, but not lecithin (142). Johnson (143) also studied the lipids in the peripheral nerve of several species. The distribution of the cerebrosides, total cholesterol and total phospholipids, and of individual phospholipids in the peripheral

nerve, resembles the proportion in the white matter rather than that in the gray matter or in the whole brain. Relatively more sphingomyelin and less cephalin and cerebroside are present in the peripheral nerve than in the white matter of the brain. It was found that, during Wallerian degeneration of the peripheral nerve of the cat, the concentration of cerebrosides, free cholesterol and sphingomyelin steadily decreased as a result of hydrolysis (144, 145). Of the phospholipids, cephalin is hydrolyzed most rapidly and lecithin most slowly, sphingomyelin having an intermediate position (146). The concentration of total lipid, total phospholipid, and cholesterol in rat brain was found to be unaffected by choline deficiency (147). McKibbin & Taylor (148) have developed a new method for the determination of sphingosine based upon chloroform extraction of the aqueous lipid hydrolysates. It is both specific and relatively quantitative. These workers (149) reported that the highest content of sphingosine nitrogen was obtained from the cerebrum (35 per cent) while it comprised approximately 17 to 18 per cent of the total lipid nitrogen in the intestine, spleen, and lung, and 8 to 12 per cent occurred in the kidney, heart, pancreas, skeletal muscle, and liver.

Preparation of lecithin and cephalin.—Sinclair (150) has described a method for preparing lecithin which is completely free from amino nitrogen and which has an excellent nitrogen:phosphorus ratio. This is done by cooling a 10 per cent solution of mixed egg-white phospholipids in absolute ethanol to −35°C. and eliminating the insoluble portions by filtration. This lecithin was more highly unsaturated than that prepared by the cadmium chloride method. A number of enantiomeric α-lecithins have been synthesized by Baer & Kates (151). α, β-Diglycerides of known configuration or racemic modifications which were composed of saturated fatty acids were phosphorylated with monophenylphosphoryl dichloride in the presence of pyridine. The reaction product was immediately esterified with choline chloride. The di-acyl-α-glycerylphenylphosphoryl choline chloride was isolated and purified as a reineckate and was later converted to the sulfate, following which the protective phenyl group was removed by catalytic hydrogenolysis. The sulfate ion was removed and the resulting hydrolecithin could be isolated and purified by crystallization from di-isobutyl ketone. The authors have prepared *l*-α, distearo-, dimyristo-, and dipalmitolecithins, whilh have a choline:phosphoric acid:fatty acid ratio of 1:1:2. The *l*-α-dipalmitolecithin was identical with the dipalmitolecithin obtained from natural sources. Hunter and co-workers (152) have described a somewhat analogous synthesis of α,γ-dimyristo- and α,γ-erucostearocephalins which was effected by treatment of the diglyceride in chloroform with phosphorus oxychloride and pyridine. followed by treatment of the acidified solution with hydrazine hydrate.

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THE CHEMISTRY AND METABOLISM OF THE STEROID HORMONES

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Introduction

Treatises on steroid chemistry in general (1) and specifically on steroid hormone chemistry and metabolism (2 to 5) have been presented in two books: the former in *Natural Products Related to Phenanthrene* by Fieser and Fieser, the latter in *The Hormones* (Volume I) edited by Pincus and Thimann. These volumes contain detailed reviews of the literature through late 1948. This review, therefore, will be confined to more recent developments, and will attempt to stress those experimental investigations of the hormonal steroids significant for the understanding of their biochemical behavior in living organisms. Studies of nonhormonal steroids will therefore be omitted, except as they offer information pertinent to this stated objective. Since the primary organs of origin of the steroidal hormones are the gonads and the adrenal cortex, we will consider in order (*a*) ovarian hormones, (*b*) testis hormones, and (*c*) adrenocortical hormones. Steroids originating from the placenta belong to the ovarian hormone group, although the recent discovery of increased adrenocorticoid excretion in pregnancy (6) suggests that the placenta may be adrenocorticosteroidogenic. It should, of course, be recognized that grouping the steroid hormones in this way involves only considerations of primary functional significance. Estrogens have been isolated from each of the steroidogenic organs, but functionally they are ovarian hormones; androgens have been isolated from testes and adrenal glands and indicated as probably minor products of ovaries, but they are primarily male sex hormones.

Recent revisions in steroid nomenclature have been described by Wettstein & Benz (7) in their 1949 review in this journal. In the preface to their book Fieser & Fieser (1) have presented extended proposals and parallel, but not entirely concordant, suggestions have been made by Heard and Prins in the former's chapter in *The Hormones* (5). The relative priority of Fieser & Fieser's proposals [first broached in 1948 (8)], has led to a more widespread adoption of their terminology. Final agreement by convention seems urgently desirable, but until it is concluded, bystanders are forced into the position of translators. In this review we shall attempt to employ the terminology of the individual papers reviewed, but shall make free use of trivial names; thus estradiol will refer to estradiol-3, 17β, the "natural" isomer formerly designated "α-estradiol"; 17-hydroxyprogesterone will similarly refer to 17α-hydroxyprogesterone, originally designated "17-β-hydroxyprogesterone," and so on.

The Ovarian Hormones

Estrogen synthesis.—Miescher (9) has reviewed the discovery and synthesis of estrogenic hormones and Dodds (10) the nonsteroidal estrogens. Miescher's review is particularly appropriate since his accomplishment with Anner (11) of a successful total synthesis of estrone through doisynolic acid intermediates [cf. (7)] has climaxed many years of effort to this end. Other approaches have been pursued by Bachmann & Johnson (12) and Johnson *et al.* (13), but these have not led to estrogenic products. Using cyclopentenyl tetralone precursors (14) Buchta and co-workers have prepared 6-oxo-1-ethyl-2-cyclopentyl-3,4-dihydronapthalene (15) which is active in 80 to 100 μg. amounts in immature rats. A new approach to partial synthesis is suggested by the preparation of an aromatic ring A in a bile acid (16).

The biosynthesis of ovarian estrogen has continued to receive attention from Claesson and his co-workers (17, 18, 19) who find decreases in cholesterol esters and residual fatty acids in the rabbit ovary following gonadotrophin administration accompanying the formation of an "estrogen precursor" (identified histologically); storage of the "precursor" is effected by pregnant mare's serum (PMS) and pregnancy urine gonadotrophin in combination but by neither alone. Intravenous PMS administration leads to a decrease in ovarian ascorbic acid which is maximal in 3 hr. (35 per cent) and slowly returns to normal (19). These changes are reminiscent of cholesterol and ascorbic acid behavior in adrenals stimulated by adrenocorticotrophin.

Estrogen metabolism.—Experimental investigations of estrogen metabolism have been reviewed by Heard (20) and Heard & Saffran (21). Segaloff (22) has considered estrogen metabolism particularly in relation to clinical problems. These papers are concerned principally with the fate of estrogen in the animal body and the processes involved in estrogen degradation and interconversion. The accepted scheme of conversion of estradiol as the principal ovarian estrogen is: estradiol (I) $\overset{(A)}{\leftrightarrows}$ estrone (II) $\overset{(B)}{\longrightarrow}$ estriol (III). That 16-ketoestrone (IV) may be an intermediate in the process (B) is suggested by the excretion of III after the administration of IV to a human subject (23). When either I or II are injected into animals or man the quantities of I, II and III recovered in the excreta ordinarily account for not more than 20 per cent of the administered steroid. In the rabbit only α-estradiol is recovered as the metabolite of β-estradiol as 40 per cent of the administered estradiol, practically exclusively in the urine with no identifiable fecal metabolite (21). In seeking to account for the missing metabolites it is suggested that substances lacking the intact estratriene skeleton are indicated since: (*a*) excretion into the bile may account for at most 35 per cent of the material (24, 25); (*b*) hydrolytic destruction in the course of extraction of the natural estrogens excreted as conjugates occurs to too small an extent to account for the "missing" steroid, [Cohen & Bates (26) find that phenolsulfatase treatment of equine pregnancy urine increases total estrogen yield by 20 per cent over the conventional acid hydrolysis], and (*c*) experiments with I^{131}-labelled iodoestradiol administered to mice suggest the excretion, particu-

larly into the feces of acidic metabolites (21, 27). The isolation of 3-desoxyequilenin from pregnant mare's urine (28) suggests a possible neutral metabolite. Niederl & Vogel (29) have prepared 2, 3-estracatechol, and finding it much less active than the parent compound (II), suggest it may represent the product of a phenolase action on natural estrogen. The finding of *m*-methoxybenzoic acid and *m*-hydroxybenzoic acid in pregnant mare's urine cannot be accountable to metabolized nutriment (30), and may represent acidic fragments from degraded estrogen.

The low concentrations of endogenous estrogens in the blood and the rapid disappearance therefrom of exogenous estrogen (20, 21) even when administered intravenously in massive doses (31), has led to considerable inquiry concerning the organs and tissues into which estrogens are localized and their significance as metabolizing centers. That the blood itself may act upon administered estrogen is suggested by the rapid disappearance of a large proportion of estrone incubated with whole blood *in vitro* (32). Since such rapid inactivation of natural estrogen occurs localization in various tissues has not been possible, but suggestive studies have been conducted with radioactive iodoestradiol in mice (21, 27). Ten hours after its administration, over 40 per cent of the radioactivity appears in the gastrointestinal tract and its contents, about 10 per cent in the urine, 5 per cent in skin and muscle, less than 1 per cent in the liver, and minor proprotions elsewhere. The estrogenolic acid, bisdehydrodoisynolic acid methyl ether, exhibits somewhat similar distributions in the rat (33, 34). The suggestion of a major excretion of estrogen metabolites into the bile and thence into the gut via the enterohepatic circulation is obvious. Pearlman & Rakoff (35) have presented evidence that all three natural estrogens are present in human pregnancy bile, chiefly as conjugates.

The appearance of significant amounts of both endogenous and exogenous estrogen in the bile has led to a number of investigations of the role of the liver in estrogen metabolism. A dual role of this organ is suggested by a number experiments: (*a*) conjugation, i.e., esterification, of the estrogens, and (*b*) conversion. Thus, Crépy (36) has found that conjugation of estrone and estradiol with glucuronic acid is effected by rabbit and guinea pig liver slices *in vitro*. Endogenous estrogen is excreted as glucuronide and sulfate (37). Synthetic estrogens (e.g. stilbestrol, hexestrol, dienestrol) administered to animals are excreted chiefly as glucuronides into the urine, but in minor amounts as sulfates (38, 39, 40). Since, however, the administration of stilbestrol glucuronide resulted in only partial recovery (19 per cent) the suggestion is clear that further metabolism of the conjugates occurs (38). That esterified estrogen may be converted is evident in the data of Pearlman & DeMeio (41) who isolated estrone (as 6 per cent of the starting material) after incubating estradiol hemisuccinate with rat liver slices; strongly acidic phenols of undetermined constitution were also present as conversion products. Several detailed studies of the estrogen inactivating system (or systems) of the liver have appeared in the past year. The chief account is given by Segaloff (22). Experiments with estradiol esters conjugated in the 3 and 17 positions indicate that the liver system acts upon ring D since 17-caprylate,

for example, is not inactivated whereas the 3-benzoate is. Various *in vitro* studies with liver mince and liver fractions suggest that the hepatic estrogen-inactivating system involves cozymase, flavine-adenine-nucleotide and cytochrome-*c*. This is in agreement with the findings of DeMeio *et al.* (42) who demonstrated a cyanide-labile system activated by nicotinamide and cozymase effective probably as a dehydrogenating system. Paschkis & Rakoff (43) have presented a detailed review of the recent work of their group. Furlong *et al.* (44) have presented data indicating a prolonged effect upon the hepatic inactivating system of CCl_4 administered to female guinea pigs; during the first 50 days of regular administration of CCl_4 estrogen excretion rises to a peak 350 times the normal, with continued administration there is a fall from this high level to levels still much above normal. Forty-five days after cessation of CCl_4 administration excretion levels fall to normal values.

The need for improved methods of estrogen extraction and more accurate analytical methods are stressed by Engel (45). Enzymatic hydrolysis of urinary estrogens [cf. (26, 37)] should improve urinary extraction yields, although pyrogallol or ascorbic acid added to urine may equally improve yields during acid hydrolysis (46). An elegant use of counter-current extraction for the quantitative separation of urinary estrogens is described by Engel, and combining this with microestimation by fluorophotometry in H_2SO_4 solution offers a remarkable means of measuring the minute amounts present, particularly in nonpregnancy urines (45). Umberger & Curtis have examined the reaction of H_2SO_4 on the various natural estrogens (47) and Boscott has suggested that the dehydration reaction involved may be more effectively carried out with concentrated formic acid (48). Boscott suggests that $\Delta16,17$ compounds are formed which are responsible for the specific fluorescences observed. Djerassi has described steroidal diphenylhydrazones having typical ultraviolet absorption spectra that may be of value in steroid analysis (49, 50). Umberger & Curtis have indicated the possibility of separating estrogenic diesters of *p*-phenylazobenzoyl from monoesters (51).

Progesterone metabolism.—Notable contributions to endogenous progesterone metabolism have been made by Hooker & Forbes as a result of their highly specific intrauterine assay for progesterone. The topical application of minute amounts of a large variety of steroids to the mouse uterus led to progestational effects only with progesterone, leading to the conclusion that practically all other gestagens are effective only after transformation elsewhere in the body to an effective specific steroid (52). Blood concentrations in female rabbits, mice, and a monkey varied from 4 to 8 μg. per ml. of whole blood; all of the activity resides in the plasma and over 90 per cent is free steroid (53). The finding of up to 3 μg. per l. in the blood plasma of cockerels and nonovulating hens suggests lesser progesterone production in the fowl and the complete absence of activity in capon plasma suggests that testis tissue is a synthetic site (54). The bound progesterone of mammalian blood plasma is inactive in the mouse test (53). When progesterone pellets are implanted in the spleen of ovariectomized mice, a large increase in bound progesterone of the blood occurs (55); this suggests that the liver "inactivation" of progesterone may involve, at least in part, the conversion of the

free steroid to this bound form. The bound progesterone levels continue for over two weeks at maximally established concentrations (55).

The principal identified metabolite of progesterone is pregnanediol-3α,-20α, but small amounts of pregnanolones are excreted after progesterone administration (56, 57). Although ethynyl testosterone is an effective progestin no pregnanediol excretion follows its administration even in large dose to men and women (56). Hooker & Forbes' finding that ethynyl testosterone is relatively inactive in the mouse test (52) suggests the need for a search for another transformation product. That the bile may be a useful source for such products is suggested by Pearlman & Cerceo's finding of a pregnanediol isomer, pregnanediol-3β,20β in pregnant cow bile (58). The isolation of 16-allopregnenol-3β-one-20-sulfate from pregnant mares' urine (59) and of allopregnanetriol-3β,16α,20β (60) suggest transformation products involving oxidative attack on Ring D. Just as with the estrogens, recovery of identifiable transformation products accounts for only a portion of administered progesterone. Marrian (57) finds in men and postmenopausal women a total urinary recovery of pregnanediol accounting for 9.3 to 16.1 per cent of progesterone, whether it is administered orally or parenterally; pregnanolone accounts for only a small additional percentage. Since pregnanediol administration itself leads to only slightly higher percentage recoveries (57) the implication of further degradation of pregnanediol is clear. Hope for more detailed analyses of progesterone metabolism is given by the preparation of 21-C^{14}-progesterone having adequate specific activity for tracer studies (61, 62), but ring-labeled progesterone would be highly desirable, especially if side-chain scission occurs in the body.

Testis Hormones

Androgen synthesis.—An approach to the total synthesis of steroidal androgen unfortunately failed when the main product from the condensation of 3,6-diketo-2,5-dimethyldecahydro-1,2-cyclopentenonaphthalene-x-α with 4-diethylaminobutan-2-one-methiodide in the presence of sodium ethoxide proved to be a derivative of 1,2-benzfluorene and not the expected androstenedione (63). Androgenic activity about 1/200 that of testosterone propionate was found in 6-(4^1-ketocyclohexyl)-$\Delta^{1,9}$-octalone-2 prepared by Wilds *et al.* (64). The introduction of a $\Delta^{9,11}$ double bond into androstenedione, testosterone acetate and 17-methyl-testosterone failed to increase the androgenic activity of these compounds nor did it confer androgenic activity on the inactive etiocholane-3α,17μ-diol (65). This is in contrast to the tripling of activity (Clauberg test) in 11-dehydroprogesterone previously reported (66). The synthesis of more potent testosterone esters has been reported by Mooradian *et al.* (67) who have used various alkoxy, alkomercapto acids and their acid chlorides to prepare a large number of the corresponding androgen esters. Holden *et al.* (68) have prepared testosterone sulfate, but no activity data are given. A curious finding by Heusser *et al.* (69) may have eventual significance in steroid biosynthesis. They found that 14-allo-17 isoprogesterone was, as might be expected from the unnatural configuration, inactive as a progestin. This compound, however, proved somewhat less ac-

tive (3/4 to 1/2) than androsterone in the capon test; even the pregnenolone isomer proved active (1/5) of androsterone. Although 14-allo-17-epitestosterone was prepared by these authors by perbenzoic acid oxidation of the side chain of 14-allo-17-isopregnenolone, its androgenic activity is not reported. The persistent suggestion that the testes of certain mammals may produce a 21-carbon androgen [cf. (70)] is recalled.

Androgen metabolism.—The metabolic fate of exogenous and endogenous androgenic steroids in animals and man has been the subject of numerous investigations and has been extensively reviewed by Dorfman (4). The general assumption has been that testosterone represents the major androgenic secretion of the testis. According to Samuels (71) it has been demonstrated in the extracts of testes of three species and where no testosterone has been isolated [cf. Haines *et al.* (72)] no other androgen has been demonstrated. Various schemes for the catabolism of testosterone have been advanced [cf. Koch (73) and Dorfman (4)]. As the result of a beautiful series of studies of enzyme action on testosterone, Samuels (71) favors the notion that testosterone is converted to Δ^4 androstenedione (I) by an enzyme system present in liver and kidney (but not in prostate, seminal vesicles, or uterus) which is activated by diphosphopyridine nucleotide (DPN). I in turn is transformed to two major isomeric products: androstanedione-3,17, (II) and etiocholanedione (III). He finds in liver and kidney a citrate-activated enzyme system which reduces the $\Delta^{4,5}$ double bond of I; the DPN-activated system converts 17-hydroxy to 17-ketosteroids and is effective also in reduction of unsaturation in ring A. Reduction of the 3-keto groups of II and III leads to the four isomers: androsterone, isoandrosterone, etiocholanol-3α-one-17, and etiocholanol-3β-one-17. He believes that these 17-ketones are further reduced to the corresponding diols. A cell-free protein concentrate of the DPN-activated system of rat liver has been prepared. Samuels (71) has given detailed data on the reduction of the $\Delta^{4,5}$ double bond and 17-ketosteroid formation *in vitro* by various livers, including the human. The data of Schneider & Mason (74) on the incubation of androsterone (IV) and etiocholanol-3α-one-17 (V) with rabbit liver slices are in accord with his view of the further conversion of saturated 17 ketosteroids, since they isolated androstanediol-3α,17α after incubation of IV and etiocholanediol-3α,17α- as well as the 3α,17β-diol after incubation of V. Since they obtained also 3,17-diones and also isoandrosterone from IV, a reversible reaction with 17-ketosteroids is suggested. Since Davis *et al.* (75) find that only 1 to 2 per cent of rabbit urinary 17-ketosteroid is androgenic (androsterone equivalent) and none in β-ketosteroid form, the significance of these *in vitro* studies with rabbit liver is not clear.

Samuels' ultraviolet absorption measurement of double bond "reduction" involves in fact the disappearance of the α, β double bond. Mason *et al.* (76) have demonstrated that the $\Delta^{5,6}$ bond of dehydroisoandrosterone (VI) is also subject to reduction *in vivo* for they isolated both androsterone and etiocholanol-3α-one-17 from the urine after administering VI to a woman following adrenal tumor excision. It is interesting that administration of VI

to the same patient before the operation led only to a small increase (about 5 per cent) in 17-ketosteroid output. Reiss *et al.* (77) report an increase in urinary α-ketosteroids following dehydroisoandrosterone administration to mental patients. Since they found pituitary gonadotrophin in these subjects depressed 3α ketosteroid excretion, they suggest that administration of VI stimulated pituitary adrenocorticotrophin secretion, which in turn might be responsible for the increase in ketosteroid output. That metabolism studies with human subjects are complicated by effects on endogenous glandular balance is suggested by such effects as the reduction in 17-ketosteroid output by estrogen administration (78, 79), although this appears to be a quantitative inhibition (78). Even psychic trauma will alter urinary steroid excretion (80).

Again it must be stressed that studies both *in vivo* and *in vitro* fail to offer a complete metabolic balance sheet for the androgenic steroids. Excretion into the bile (43), particularly in certain species, e.g. the cow (81), may account in part for the missing metabolites in *in vivo* experiments, but a search for further products is clearly called for. The recent finding of Δ^{16} androstenol-3α as a glucuronide in human male urine (82) suggests that other types of steroid should be sought. Tracer studies with C^{14}-labeled androgen should give clues to nonsteroidal catabolites.

Urinary ketosteroids in man.—Since the urinary 17-ketosteroids derive at least in part from adrenocortical precursors, their discussion in this section is somewhat ambiguous. Nonetheless a number of studies of 17-ketosteroid excretion involve aspects of gonad function. For example, low 17-ketosteroid output with qualitative alteration of the excretion pattern have been reported for men with gynecomastia (83). Wolfson *et al.* (84) find markedly diminished 17-ketosteroid excretion in men with gout, but no clinical signs of diminished androgen. The elevated 17-ketosteroid output in subjects with ankylosing spondylarthritis (85) probably relates to adrenocortical activity. The variations in 17-ketosteroid excretion with age have been the subject of a number of papers (86, 87, 88). Generally, increases in pubertal children (86) contrast with decreases in ageing men (88). Whether these changes, involving either age or various pathologies, signify glandular secretory changes or alterations in steroid metabolism is unknown.

Improved analytical methods for urinary ketosteroid analysis have been the subject of some discussion. Engel (45) has reviewed current colorimetric methods. Jones & Dobriner (89) have presented a detailed account of infrared spectrophotometric analysis. The elegance of this method both for qualitative and quantitative analysis is obvious and its increased application may be expected to yield much new information. The application of the Pettenkofer reaction to the identification of 3β, Δ^5 urinary steroids has been described by Lorenz (90); Lange *et al.* (91) find that such steroids may be converted to colored compounds by $HClO_4$ or HPF_6 in acid. Miescher & Kägi (92) have reexamined the color reaction of 17α-hydroxy steroids in dilute acetic acid-sulfuric acid solution with bromine in glacial acetic acid. A hydroxide reagent for the histochemical demonstration of active carbonyl

groups has been examined by Seligman and co-workers (93, 94), and applied to the testis, the adrenal cortex, corpora lutea, and placenta.

Adrenal Cortex Hormones

The discovery of the antiarthritic effect of 11-dehydro-17-hydroxy-corticosterone (cortisone) and of pituitary adrenocorticotrophin (ACTH) by Hench, Kendall, Slocumb & Polley (95) has enormously stimulated studies of the chemistry and metabolism of adrenocortical steroids. Much of this work is in process and any review at this time is bound to be fragmentary. The need for large quantities of these substances as medicaments has incidentally remedied a situation that has plagued research workers for many years, namely the scarcity of adrenocortical steroids which has effectively blocked studies of their metabolism.

Adrenocorticosteroid synthesis.—Fieser & Fieser (1) have reviewed the synthetic chemistry of adrenocortical steroids [see also Laland (96)]. The involved chemistry has led to attempts at partial synthesis, particularly of 11-oxygenated, 17α-hydroxy corticosteroids possessing biological activity. The most used starting material is desoxycholic acid, since it possesses an oxygen function in ring C and has the proper ring configuration. Oxidation of the side chain at C_{17}, the building up of the needed hydroxyacetone at C_{17}, conversion of the 12-oxygen to an 11-oxygen function and oxidation to the 3α, β unsaturated ketone have all been effected in a brilliant series of syntheses by Reichstein, Kendall, Sarett and co-workers. The limited supply of animal bile acids and the complexities of the syntheses therefrom has led to the suggestion of other precursors. Thus, Marker has examined butogenin and ricogenin as plentiful plant precursors (97, 98). From the former he obtained by oxidative degradation an allopregnanetrione-3,12,20, but Wagner *et al.* (99) find their product differs from allopregnantrione-3,12,20 synthesized from desoxycholic acid and so question the suitability of butogenin as a precursor. The 16-dehydropregnen-5-ol-3-dione-12,20 prepared from ricogenin by Marker (98) appears to be a desirable intermediate. Lardon (100) has undertaken various partial syntheses using periplogenin as starting material and has obtained progesterone, 11-desoxycosterone, 14-oxo-14-iso-11-desoxycorticosterone and other products, but no likely intermediate to cortisone synthesis.

Sarett has continued his studies of stereoisomeric substituted 11-keto-20 hydroxypregnanes (101), and has described the preparation of pregnan-17α 21-diol-3,11,20-trione-21 acetate (102). Julian and co-workers have described the synthesis of intermediates leading to the production of 17α-hydroxyprogesterone and 17α-hydroxy-11-desoxycorticosterone (103, 104). Gallagher and co-workers have published in detail on various approaches to cortisone synthesis. The conversion of 3α,12β-dihydroxy-11-ketocholanic acid to 3α-hydroxy-11-ketocholanic acid has been described (105) as well as related 3α,11α-dihydroxy steroids (106). Using a different approach than that of Julian *et al.*, Gallagher and co-workers have prepared various 17α-hydroxysteroids. Instead of preparing 16,17-oxido precursors, the latter group has employed the enol acetates of 20-ketosteroids (107), arriving by

various oxidative procedures to 17-hydroxyprogesterone and 17-hydroxy-11-desoxycorticosterone (108). MacPhillamy & Scholz (109) have described the use of the Hofman degradation for the introduction of the Δ^{11} double bond into various steroids. Khan & Linnell (110) have continued attempts to prepare stilbene derivatives having corticosterone-like activity.

Modes of biosynthesis of corticosteroids have been suggested by the recent work of Hechter and collaborators with the isolated perfused adrenal. Employing a perfusion apparatus recently described (111), Hechter has observed the increased production of formaldehydogenic neutral lipid and concomitant glycogenic activity after the addition of ACTH to the perfusion medium (112). Perfusion of the isolated beef gland with 11-desoxycorticosterone led to the production of corticosterone (113). This ability of the gland to effect 11-oxygenation is not confined to 11-desoxycorticosterone since 11-hydroxyandrosterone has been isolated after perfusion with androsterone and 17-hydroxycorticosterone after perfusion with 17α-hydroxy-11-desoxycorticosterone; 11-oxygenation of other steroids (e.g., progesterone, 17α-hydroxyprogesterone) is indicated (114). Since this 11-oxygenation is effected in the absence of ACTH, and is not materially enhanced by the addition of ACTH to the perfusate, this step in the biosynthesis of typical adrenocorticosteroid is obviously beyond ACTH control. Since perfusion with precursors not hydroxylated at C_{17} has led in no instance to the production of 17-oxygenated steroids either in the presence or absence of ACTH, it is clear that this function of the adrenal cortex is performed in a reaction independent of that effecting the 11-oxygenation. The disappearance of a large proportion of adrenal cholesterol during the period of secretory activation by either stress procedures or ACTH administration has suggested that cholesterol is the precursor of adrenocortical steroids [cf. Long (115), Sayers & Sayers (116)]; the studies of Hechter *et al.* suggest ACTH action may involve either the degradation of the cholesterol side chain and/or the condensation of a suitable ketol side chain at C_{17} to a C_{19} precursor. Alternatively ACTH may promote synthesis from triose precursors. Since adrenocortical tissue *in vitro* produces cholesterol from C^{14}-labelled sodium acetate (117), its capacity to produce adrenocortical hormones demands investigaltion; the almost geometric condensation of acetate into the cholesterol molecule [Block (118)] suggests that a 19-carbon, or 21-carbon condensate is not improbable. Discussions of adrenocortical hormone synthesis are presented by Kendall (119), Pincus (120), and Block (121) in the first Macy Foundation Conference on the adrenal cortex. The suggestion that energy for adrenocortical hormone secretion is derived from phosphate bond energy arises from the studies of Gemzell (122) with radiophosphorus. An increased uptake of P^{32} by ACTH-stimulated adrenals is also reported by Reiss (123).

Adrenocorticosteroid catabolism.—As indicated previously, studies in this field have been limited by the nonavailability of most adrenal cortex steroids. Urinary excretion studies in human subjects have given rather fragmentary information to date. Recent data have been reviewed by Sprechler (124). Both urinary 17-ketosteroids and corticosteroids in man are considered as metabolites of adrenocortical precursors. This is clearly indicated by the

increased excretion of each following ACTH administration (125, 126, 127). Activation of the pituitary-adrenal system by stress procedures also effects increased output of these steroid types, not only in the urine (128) but also into the sweat (129, 130). Nonetheless the secretory precursors of each are still being sought. Surprisingly small increases in 17-ketosteroid output occur after the administration of cortisone whereas urinary corticoids increase notably in amount (119, 127) even in Addison's disease patients. Adrenal extract administration is followed by prompt increase in 17-ketosteroid output (126, 128). In seeking for a unitary concept of adrenocortical secretion (e.g. the secretion of an 11-oxygenated 17α hydroxy α-ketol such as cortisone or 11-hydroxycorticosterone) Thorn and Forsham (127) are faced with the possibility of an adrenal 17-ketosteroid precursor produced in large amount after ACTH administration [cf. Albright *et al.* (131)]. It is clear from the brilliant analytical work of Dobriner, Lieberman and collaborators [see (5) and (89)] that the total 17-ketosteroids of urine represent a large group of substances and that the specific identification of adrenocortical metabolites is necessary for the understanding of the metabolic significance of urinary 17-ketosteroids. Similar considerations undoubtedly apply to the urinary corticosteroids since the hormonally active corticoid is always less than the reducing lipid (5, 125) or the formaldehydogenic steroid (132) of urinary extracts. Dobriner and co-workers (89), have considered 11-oxygenated steroids, such as 11-hydroxyandrosterone, as specific adrenocorticosteroid catabolites, and it is noteworthy that this particular compound appears to be excreted largely unchanged after administration to an Addisonian patient (127). Nonetheless, the finding of Hechter *et al.* (114) that the adrenal may effect 11-oxygenation of androsterone and other nonadrenal steroids reopens the question of the probable origin of 11-oxygenated compounds.

Studies of tissue systems presumed to act upon adrenocorticosteroids have been scant. Green (133) finds that desoxycorticosterone effects are much diminished when pellet implantation of this compound is made in the spleen, indicating a hepatic inactivating system. That this inactivation may involve protein binding is suggested by the finding of such an effect upon progesterone by Forbes & Hooker (55). Samuels (71) found that rat liver *in vitro* destroys the α, β unsaturation not only of testosterone but also of progesterone and by analogy a similar effect on ring A of α, β unsaturated corticosteroids is implied.

Three analytical methods for urinary corticosteroids have been compared by Romanoff *et al.* (132); these are the determination of reducing lipids by two methods and of formaldehydogenic lipid. Engel (45) has reported similar comparative results. Pincus & Romanoff (134) have improved extraction yields by continuous extraction under mild hydrolytic conditions and effected a separation on silica gel. Zaffaroni, Burton & Keutmann, following their studies with 17-ketosteroids (135), have examined paper partition chromatography of adrenocorticosteroids (136) and have found that satisfactory partitioning may be effected by the solvent systems benzene: formamide and toluene: propylene glycol.

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THE CHEMISTRY OF AMINO ACIDS AND PROTEINS[1]

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Chemistry of the Amino Acids

Configurational relationships.—Wolfrom, Lemieux & Olin (1) have degraded natural D-glucosamine to natural dextrorotatory alanine. The amino group is attached in glucosamine to carbon 2 which is configurationally related to D-glyceraldehyde but the aldehyde group of the sugar has become the methyl group of the amino acid and the configuration is, in effect, reversed. The correlation of L-glyceraldehyde with dextro alanine is thus established. Since the latter is related to levo serine, the two standards of reference, glyceraldehyde used in the carbohydrates and serine, proposed for amino acids, are directly correlated. The configuration of carbon 2 of glucosamine is D_g but L_s, the subscripts referring to the glyceraldehyde and serine standards respectively.

Reactions of amino groups.—The velocity of deamination of amino acids in the Van Slyke nitrous acid method has been studied by Cristol and coworkers (2 to 6). The velocity constants, calculated as first order in the presence of excess nitrous acid, differ by more than two fold from one another. The velocities were increased about 100 per cent in the presence of 2 moles of potassium iodide per amino group, probably as a result of specific reaction with the latter. In the action of nitrous acid on glycine and polyglycines, Viscontini (7) has demonstrated the formation of oxaloglycine derivatives in place of the expected hydroxyacetylglycine derivatives. The oxidation which is involved may account for the excessive amounts of nitrogen which are formed from glycyl compounds.

Leonis (8) has measured the rate of liberation of hydrogen ion and the equilibrium in the reaction between amino acids and carbon bisulfide. This reaction which leads to dithiocarbamates, is very rapid for imino acids and thiols, moderately so for amino acids. Imidazole, guanidine, indole and amide groups do not react. Methods are outlined for the qualitative and quantitative estimation of thiol, amino, and imino groups with the aid of this reagent. The 2′-mercapto-thiazolones described by Heilbron (see below) are anhydrides of the dithiocarbamates formed in the reaction.

Ehrensvärd (9) has described the use of the phenylthiocarbonyl group as a blocking agent, analogous to the well known carbobenzoxy group. The

[1] This review covers the period from December 1948 through November 1949. The reviewers have intentionally omitted or treated in a cursory fashion those studies which appeared to recommend themselves to the attention of the writers of other chapters in this volume. Much material related to the subject of this chapter is considered in the chapters on enzymes, hormones, muscle, blood plasma and serum, immunochemistry, antibiotics and paper chromatography.

group is removed from the dipeptide by the action of lead acetate in solution rather than by reduction. Zeile (10) suggests the use of *p*-(benzeneazo)-phenylisocyanate and Reich (11) proposes the use of *p*-(benzeneazo)-phenylsulfonyl-chloride to produce colored derivatives of the amino acids for chromatography.

Degradation of α-amino acids to aldehydes and ketones, ordinarily accomplished analytically by ninhydrin, can be carried out with the less expensive reagent, peri-naphthindan-2,3,4-trione, which has the further advantage of being recoverable (12, 13, 14).

Karrer and co-workers (15 to 18) employ lithium aluminum hydride to reduce α-amino carboxylic esters to amino alcohols. These derivatives are sometimes useful in establishing configurational relationships.

Synthetic Chemistry

Heilbron (19) has reviewed the work in his laboratory on 5-aminothiazoles and 5-amino-2′-mercaptothiazoles. The latter are crystalline compounds readily formed by the action of carbon bisulfide on amino nitriles. They are converted to α-amino acids by dilute mineral acid. Aldehydes and ketones condense directly with 2′-mercaptothiazolone at the 4 position. Reduction and hydrolysis of the resultant derivatives gives good yields of α-amino acids. Amino acid esters react with 2′-mercaptothiazolone in the presence of triethylamine, adding glycyl units in a manner controlled by the addition of the triethylamine. Tetraglycylglycine ester has been synthesized in this way. An unusual feature of the reactions which are involved is that they take place smoothly at room temperature. A similar reaction occurs between 4-substituted mercaptothiazolone and amino acids to yield a series of peptides. Bailey (76) has outlined the use of N-carboxy amino acid anhydrides (2,5-oxazolidinediones) in controlled peptide formation. An amino acid ester and the anhydride react at 0° in the presence of triethylamine to give an unstable intermediate which on heating at 30 to 40° decomposes to carbon dioxide, triethylamine and a peptide ester. A new amino acid may be added to this peptide ester by repetition of the reaction. Syntheses of pentapeptides have been carried out, but details are not given.

Acrylic aldehyde and methyl mercaptan combine smoothly to give methylthioproprionaldehyde in the presence of tertiary amines (20, 21) or copper methyl mercaptide (22). This aldehyde is readily converted to methionine by using the Strecker synthesis (yield 27 per cent) (20, 21, 22) or the Bucherer hydantoin synthesis (yield 50 per cent) (22). The hydantoin intermediate in this synthesis can also be prepared from 5,2′-chloroethyl hydantoin and sodium methylmercaptide (23). The yield of methionine is reported as 70 per cent. Triceri & DeBarieri (24) describe a synthesis of methionine from β-chloroethyl-methyl-thioether and formylaminomalonic ester followed by hydrolysis.

The reaction of pyruvic acid with acetamide gives α-acetamidoacrylic acid which reacts with thioacetic acid to give S,N-diacetyl cysteine [Schöberl

& Wagner (25), Farlow (26), Behringer (27)]. Elliott (28) describes the synthesis of cystine starting with DL-N-thiobenzoylserine methyl ester. The latter is converted to a phenylthiazoline by thionyl chloride. The thiazoline is hydrolyzed and oxidized to cystine.

Using isocrotonic acid as a starting point, Pfister *et al.* (29) have prepared a low melting α-bromo-β-methoxybutyric acid by addition of the elements of methyl hypobromite. On amination this gave 55 per cent of DL-threonine. The higher melting bromo acid produced by West, Krummel & Carter (30) from crotonic acid gives mostly allothreonine on amination (31). The benzoyl derivatives of threonine and allothreonine are converted to different oxazoline derivatives by thionyl chloride but the derivatives are interconvertible by the action of alkali, both of them giving 27 per cent of threonine after alkaline hydrolysis (32). Attenburrow, Elliott & Penny (33) describe the direct preparation of the oxazoline from sodium hippurate and acetic anhydride. Carter & Zirkle (34) describe a synthesis of allothreonine by amination of α-bromo-β-hydroxybutyric acid, produced from crotonic acid by passing air and bromine through it. The application of the oxazoline conversions described above would yield threonine.

Syntheses of DL-tryptophane, DL-methionine, DL-glutamic acid and DL-lysine, starting with a single intermediate, γ-acetamido-γ,γ-dicarbethoxybutyraldehyde, are described by Warner & Moe (35, 36). The phenylhydrazone forms indolyl compounds which are converted to tryptophane by boiling in sulfuric acid, and is reduced to an ornithine precursor by reduction on Raney nickel catalyst. On oxidation with permanganate, the intermediate gives DL-glutamic acid precursor while lysine is formed through the cyanhydrin of the intermediate.

Turba & Schuster (37) and Kurtz (38) have synthesized arginine from ornithine using cupric ion to protect the α-amino group. The general utility of this method of protection by chelation is shown (38) by the synthesis of numerous derivatives of lysine and ornithine at the omega position.

The use of enzymes in the resolution of various amino acids is illustrated by the asymmetric hydrolysis of DL-tryptophane methyl ester (39) and DL-methionine isopropyl ester (40) by pancreatic enzymes, and of chloroacetyl-DL-alanine and other acylated amino acids by hog kidney and beef pancreas enzymes (41, 42, 43). The asymmetric synthesis of the anilides of carbobenzoxy derivatives under the influence of papain has been used by Dekker & Fruton (44) to resolve methionine and by Hanson & Smith (45) to resolve tryptophane. Polglase & Smith (46) point out that, in the preparation of peptides, the coupling of an optically active component with a racemic component often offers the opportunity to resolve the product into single isomers of the two diastereoisomers.

Protein Analysis

Amino acids.—With the development of rapid and precise chromatographic and isotopic (47, 48) micromethods for the analysis of mixtures of amino

acids, the analytical chemist is in process of recapturing from the microbiologist his proper responsibility for the systematic analysis of proteins. Moore & Stein (49) have brought the starch column to a high degree of refinement as an analytical instrument and have reported (50) essentially complete chromatographic analyses of β-lactoglobulin and bovine serum albumin. Using a combination of chemical and microbiological methods, Velick & Ronzoni (51) have given a substantially complete accounting of the amino acid residues of crystalline aldolase and glyceraldehyde phosphate dehydrogenase. They also report analyses of serum albumin and insulin, adding confirmation to the feeling that the compositions of these two proteins are now closely defined. Equally comprehensive analyses have been made by Gordon *et al.* (52, 53) of whole casein and of the α- and β-casein fractions of Warner. The three proteins differ significantly in composition, particularly in the amounts of proline, tyrosine and tryptophane which they contain. There is less than 0.1 per cent of cystine in β-casein. Extensive but incomplete analyses of whale myoglobin (54), salmine and clupeine (55, 56), lysozyme (57), myosin and tropomyosin (58) have been reported.

Bowes & Kenten (59) have combined analytical data from various sources in an accounting of 99 per cent of the nitrogen of collagen. The results are correlated with titration curves of the protein. Microbiological assays have been made of the amino acids in six types of scleroprotein (60). Some significant differences are noted. Differences have also been found, as a result of paper chromatographic comparisons, of hydrolysates of collagen, elastin and reticular tissue (61) and of the cortex and medulla of wool (62). The great value of paper chromatography for the rapid delineation of the general amino acid patterns of proteins is well recognized. The method is reviewed in another chapter of this volume (pp. 517 to 542).

Jacobsen (63) has performed a useful service in reminding the analyst that heavy metals in the hydrochloric acid used to hydrolyse proteins may, by catalysing the deamination of amino acids, lead to significant analytical errors.

Products of partial hydrolysis.—One advantage of the newer methods of amino acid analysis is that they are readily adaptable to the estimation of the simpler peptides. If the terminal residues in the chains of a native or derived protein are first tagged (64, 65) with an easily identifiable label and the protein is then subjected to partial hydrolysis and the labelled peptides are identified and estimated, valuable information can be accumulated on the sequence of amino acids in the chains of a protein. Sanger has developed this method using dinitrofluorobenzene as the label and has applied it, in particular, to insulin (66). An important consideration is that the label which is attached to the end groups should be resistant to acid hydrolysis. Sanger's reagent is fairly satisfactory in this respect, but is not ideal.

Consden, Gordon & Martin (67, 68) have sought to identify the amino acids directly attached to cystine and to the dicarboxylic acids in wool by isolating and identifying the acidic peptides, including those of cysteic acid,

which are obtained on partial hydrolysis. Employing ion exchange separations followed by ionophoresis, they have succeeded in identifying some 19 peptides of aspartic and glutamic acids and a number of peptides of cysteic acid.

In Virtanen's laboratory, the study continues of the nature of the plasteins formed from a number of proteins by treatment with pepsin under controlled conditions (69, 70, 71). Earlier estimates of the average molecular weight of these products have been revised upwards. They are now considered to be mixtures of polypeptides averaging about 4,000 in molecular weight.

Analytical methods.—Methods of estimating the total nitrogen of a protein continue to come under scrutiny. Hiller, Plazin & Van Slyke (72) believe that estimations in agreement with those obtained by the method of Dumas can be secured, by Kjeldahl's method, only if a mercury catalyst is used. A modification of Brüel's micro Kjeldahl procedure, suitable for 1 to 10 μg. nitrogen, has been described by Shaw & Beadle (73).

Several investigators (74, 75, 172) have examined the variations which occur with different proteins in the extinction coefficient in the biuret reaction. This quantity undoubtedly varies with the percentage of peptide nitrogen in the protein but there is some evidence that copper may combine with sites other than the peptide linkages (74) and Emmrich (75) suspects that changes in the configuration of the protein may affect the color. Stiff (77) has described a microadaptation of the biuret reaction in which the copper bound to the protein is estimated with the aid of diethyldithiocarbamate. The same reagent has been used by Woiwod (78) in an adaptation of the reaction of copper with α-amino compounds to the microanalysis of mixtures of amino acids and peptides. This method should be valuable in the analysis of paper chromatograms.

Friedman (79) reviews the methods available for the estimation of sugars in proteins and devises procedures based on the orcinol and carbazole reactions. Quantitative results depend on a qualitative knowledge of the sugars which are present.

There have naturally been many attempts to extend chromatographic methods to the separation of individual proteins in mixtures. Tiselius (80, 81) has had some success in separating proteins on silica gel and on paper by development with salt solutions which are rather concentrated but are well below the concentrations required to salt-out the proteins concerned. Franklin & Quastel (82) have recently published reproductions of paper chromatograms which show some degree of resolutions of protein mixtures while Mitchell, Gordon & Haskins (83) have claimed some success in the separation of mixtures of enzymes using a pile of filter papers in place of a strip. An interesting front analysis of a mixture of egg-white proteins on a column of a cation exchange resin has just been published by Sobel, Kegeles & Gutter (84). The extent to which the observed separation of boundaries was due to ionic interchange with the proteins is not clear from the brief description given.

THE ISOLATION AND CHARACTERIZATION OF PROTEINS

Proteins of the egg.—There does not appear to be any difference between the electrophoretic patterns of the proteins in the three layers of the white of the hen's egg (85). On the other hand, the patterns of the egg white of different birds vary characteristically. Bain & Deutsch (86) have examined the white of the eggs of 13 species of birds and find that the most inconstant component is the albumin. It varies considerably both in mobility and in the relative amounts of the A_1 and A_2 components. Meanwhile, Cann (87) finds further evidence of a small amount of a third component in crystalline hen ovalbumin.

The characterization of plakalbumin has been materially advanced in the laboratory of Linderstrom-Lang during the past year. Plakalbumin is the protein which may be crystallized from a solution of crystalline ovalbumin after mild digestion with an enzyme from *B. subtilis* (88, 89). The derived protein retains the property of heat coagulability (90) but is about six times as soluble in solutions of ammonium sulfate as is the parent protein (91). The molecular weight (osmotic pressure) is $44,700 \pm 100$, compared with 45,000 for ovalbumin (92). About six nitrogen atoms are lost in the transformation of ovalbumin to plakalbumin (90) but the phosphorus content is not changed. The derived protein exhibits electrophoretic inhomogeneity similar to that of the ovalbumin from which it was prepared. The two proteins show a constant difference in mobility at pH values below 7, the isoelectric point of plakalbumin being 0.14 units pH higher than that of ovalbumin over a range of ionic strengths of acetate and of acetate-chloride buffers (93).

Bain & Deutsch (94) have isolated an amorphous preparation of conalbumin containing rather loosely bound flavin. It is electrophoretically homogeneous under restricted conditions but shows evidences of heterogeneity at low ionic strength at the isoelectric point and after exposure to a low pH (cf. 95). The molecular weight (sedimentation-diffusion) is 87,000. Schade, Reinhart & Levy (96) have made the interesting observation that carbon dioxide is necessary for the formation of the salmon-pink complex of iron with conalbumin and with siderophilin. To explain this, Fiala & Burk (97) suggest that the color may depend on the formation of a cyclic hydroxamic acid grouping with which the metal coordinates.

An interesting phosphoprotein, phosvitin, has been isolated from egg yolk by Mecham & Olcott (98). It represents about 7 per cent of the protein of the yolk but contains 60 to 70 per cent of the phosphoprotein phosphorus. The high phosphorus content (10 per cent) is in approximate stoichiometric agreement with the high content of serine (32.5 per cent). Phosvitin is homogeneous in the ultracentrifuge (mol. wt. 21,000) but shows some boundary spreading on electrophoresis. There is evidence of aggregation in the presence of Mg^{++}. Shephard & Hottle (99) find that the livetin of egg yolk has three components which are related in their mobilities to the albumin and two globulin components of the serum of the hen.

Many biological fluids contain an agent which inhibits the agglutination of red cells by influenza virus. The richest source of this agent is egg white. Lanni *et al.* (100) have prepared a material 60 times as active as egg white. It is rich in carbohydrate, forms very viscous solutions and may be identical with ovomucin (ovomucoid-β).

Proteins of milk.—Careful redeterminations of the sedimentation and diffusion constants of β-lactoglobulin have been reported by Cecil & Ogston (101). Their values differ from those in the literature but they combine to give a molecular weight of 35,400 in excellent agreement with the value arrived at by Senti & Warner (102). Halwer & Brice (103), using the light scattering method of Debye and a new design of turbidimeter (104), compute a slightly lower molecular weight of 33,700.

The heterogeneity of β-lactoglobulin continues to attract interest. The crystalline fractions of varying solubility which have been obtained by McMeekin and his associates (105) have been further examined. The fractions, like the parent protein, will crystallize with bound dodecylsulfate (106) the crystals differing from one another and from β-lactoglobulin in solubility and in mobility. No difference has been found, in the Carlsberg laboratory (63), between the mobilities, solubilities, titratable groups and amide groups of samples of β-lactoglobulin prepared from the same milk by the respective methods of Palmer and of Sorensen.

Proteins of muscle.—The relations of the various proteins of muscle to the contractile process will be reviewed elsewhere in this volume (pp. 371 to 388). In this chapter we will be content to draw attention only to a number of crystalline proteins which have recently been isolated from muscle. Several of these are enzymes while others, prepared respectively by Bailey (58), Dubuisson (107), Bourdillon (108), and Distèche (109) are probably related to the contractile elements. The preparation of Bailey has been named tropomyosin in the belief that it may be the prototype from which the large myosin molecule is assembled. Tropomyosin is prepared by the extraction of alcohol-ether dried muscle with relatively strong salt solutions. Once extracted, however, the protein is soluble in water to form very viscous solutions. Although electrophoretically homogeneous, solubility tests give some evidence of heterogeneity. The molecular weight (sedimentation-diffusion) is reported to be 92,700 (110) but the value derived from measurements of osmotic pressure has been found to vary from 51,000 to 135,000 as a function of the ionic environment. (111). Since the minimum molecular weight, based on the presence of three histidine residues, is 54,500, it is suggested that the lowest value derived from osmotic pressures best represents the unpolymerized molecule. The amino acid composition (58) and the x-ray diffraction patterns (112) are both quite like those of myosin. Electron microscopic examinations of deposits from aqueous solutions of tropomyosin have been reported (112).

Snellman and his associates (113) have examined the ultracentrifugal behavior of the crystalline myosin of Szent-Györgi and (114) have compared

it with that of myosin prepared by the method of Greenstein and Edsall. Neither preparation is homogeneous. The molecular weight of the crystalline product is found to be 1.5×10^5. The isoelectric point in potassium chloride is 5.4 but rises to values as high as 8.7 when potassium is replaced by calcium or magnesium (115, 116).

Najjar (117) has crystallized phosphoglucomutase from muscle in an amount corresponding with 2 per cent of the muscle protein. Petrova (118) has prepared amylose isomerase and suggests that it may be identical with globulin-X of muscle. Arginine phosphorylase has been crystallized from extracts of crayfish muscle by Szorenyi, Dvornikova & Degtyar (119) and new methods of crystallizing myoglobin have been described (120, 121). Bowen (120) finds the iron content of horse heart myoglobin to be 0.323 per cent rather than the commonly accepted value of 0.345 per cent. The revised value corresponds with a molecular weight of 17,300 in close agreement with computations from sedimentation and diffusion constants. Bowen (122) also records extensive spectroscopic data (450 to 1,000 Å) on myoglobin, and on oxy-, carbonyl- and met-myoglobin.

Miscellaneous crystalline proteins.—Drabkin (123) describes a convenient method for the preparation of crystalline human hemoglobin on a large scale and Kubowitz (124) reports on the crystallization of this protein with the aid of ammonium sulfate. Further details have been published of the procedure used by Wilhelmi, Fishman & Russell (125) to crystallize the growth hormone of the anterior pituitary. Sedimentation and diffusion constants lead to a molecular weight of 49,200 (126) which may be compared with the value of 44,000 reported by Li & Moskowitz (127) for their amorphous but homogeneous product.

The interrelations of the various chymotrypsins constitute an interesting problem in protein chemistry. Schwert (128) has studied the ultracentrifugal behavior of chymotrypsinogen and of α- and γ-chymotrypsin while Schwert & Kaufman (129) have examined δ-chymotrypsin and further observations on chymotrypsinogen B and chymotrypsin B have been reported from the laboratory of Laskowski (130). The differences in enzymatic activity of the various proteins are beyond the scope of this review but a few purely chemical differences are of interest. It is suggested that the properties of δ-chymotrypsin are consistent with the view that it is an intermediate between chymotrypsinogen and α-chymotrypsin (129). Ultracentrifugal behavior suggests that the α- and γ-enzymes associate to dimers to an extent determined by the concentrations of the protein and of the hydrogen ion. The sedimentation constant of chymotrypsinogen is 2.7; the constants for the α- and γ-enzyme dimers are 3.5 and 3.2 respectively (128). A note appended to the last paper quotes unpublished sedimentation data from other laboratories. The isoelectric points of chymotrypsinogen B and chymotrypsin B are reported to be 5.2 and 4.7, in sharp contrast with those of the corresponding α-enzymes which are 9.1 and 8.3. These striking differences either reflect pronounced differences in composition or are indicative of strong specific anion binding similar to that noted by Velick (131) in the case of aldolase.

Tauber, Kershaw & Wright (132) have prepared from soybean a crystalline trypsin inhibitor which is similar to the product obtained by Kunitz, except that it is stable to heat. A new method of preparation of a crystalline inhibitor from the pancreas has also been described (133).

Crystalline preparations of catalase have been obtained from a bacterial source (134) and from human erythrocytes (135). In the preparation from the former source, effective use was made of lysozyme to liberate the enzyme from the bacterial bodies. The two catalases were indistinguishable in ultracentrifugal behavior (136).

Schwimmer & Balls (137) have published further details of the preparation and properties of the crystalline α-amylase of germinated barley. Two new crystalline proteins from vegetable sources have been described. One, prepared from dried beans (*Phaseolus vulgaris*) by Bourdillon (138) by alcohol fractionation, accounted for 16 per cent of the nitrogen of the bean. The other was obtained by Carpenter & Smith (139) from tomato juice and was found to have the rather low isoelectric point of 3.43.

Attention may conveniently be drawn at this point to an extensive survey [Danielsson (140)] by sedimentation analysis of the proteins of the seeds of 34 species of Leguminosae and of nine species of Graminae. The author distinguishes two types of seed globulin. The γ-type derive from the embryo and have molecular weights of the order of 210,000. The α-globulins (mol. wt. circa 29,000) come from the residual fraction. Two well-defined globulins, vicilin (mol. wt. 186,000) and legumin (mol. wt. 331,000) were isolated from *Pisum sativum* (140). They are immunologically related, but differ in amino acid composition. Their counterparts are present in most Leguminosae (141).

The Proteins of Blood Plasma

In this report last year attention was drawn to the attempt in Kirkwood's laboratory to fractionate protein mixtures by a combination of horizontal electrophoretic transport and vertical convective transport. The apparatus is so constructed that components move from an upper to a lower reservoir at rates dependent upon their individual mobilities. Preliminary studies (142) were successful in preparing 97 per cent pure γ-globulin from diphtheria antitoxin pseudoglobulin in five successive runs. The method has been further refined and an improved apparatus has been designed (143). If the isoelectric points of the components are sufficiently far apart, an effective method of operation is to adjust to the pH of each isoelectric point in turn and so to immobilize each component successively in the upper reservoir. For the fractionation of a mixture of interacting proteins such as the γ-globulins it has proved possible, by judicious control of pH, ionic strength, field strength, and duration of the run, to achieve high efficiency in separation. Thus (144, 145), from bovine γ-globulin, eight fractions have been obtained of varying mobility and with mean isoelectric points ranging from 7.31 to 5.74.

The classical methods of the Harvard laboratories continue to be developed systematically toward the separation of the multitude of physiologically distinguishable protein components present in the various commercially

available primary plasma fractions. Fibrinogen, which is difficult to purify because of its lability, is associated in Fraction I of human plasma with a nonclottable cryoglobulin of rather similar solubilities and also with an antihemophilic globulin. Morrison, Edsall & Miller (146) have been able to remove the cryoglobulin by taking advantage of its low solubility in the cold under controlled conditions of pH and ionic strength. The authors state that the purification of the antihemophilic protein has not been advanced by this work.

Further purification of Fraction IV-4 by Surgenor *et al.* (147) has given a series of subfractions rich in cholinesterase, carbohydrate-rich α_2-globulins, β_1-metal-combining globulin, and albumins. Oncley and his associates (148) have developed methods for the large scale fractionation of Fractions II and III using careful control of pH, temperature, and concentrations of salt and of protein. The following components have been effectively purified: the γ-globulin antibodies in II, the isoagglutinins in III-1, the prothrombin in III-2, the plasminogen in III-3, and the β_1-lipoproteins in III-0. A table is given (148) of the distribution of components in the various subfractions.

Hess & Deutsch (149, 150) continue to refine the practical methods of Deutsch & Nichol (151, 152) for the economical production from hyperimmune sera of fractions rich in antibodies. The main γ-globulin fraction may be further separated into γ_1 and γ_2 fractions which are essentially monodisperse from the molecular kinetic point of view, although they exhibit some degree of electrophoretic inhomogeneity. On the basis of their latest work (150), the authors find reason to hope that it may be possible by chemical means to separate individual antibodies from one another.

A copper-containing protein named caerulo-plasmin has been isolated from serum by Holmberg & Laurell (153). It contains most of the copper of serum and, although similar to hemocuprein, does not appear to be identical with it. The molecule (mol. wt. 151,000) contains eight atoms of copper. Abrams, Cohen & Meyer (154) describe a cryoglobulin prepared from the serum and lymph nodes of a case of lymphosarcoma. This protein differs from any known component of normal serum. Studies on globulin fractions obtained by salting-out procedures include those of Silva & Rimington (155) and Biserte & Masse (156), while an immune fraction has been prepared from bovine colostrum by McDougall (157). Doladilhe & Legrand (158) claim that a filtrate free from antigenicity may be prepared from serum by a simple precipitation with lead chloride. All of the antigenic properties may be recovered from the precipitate. Finally attention is drawn to the use by Reid & Jones (159) of ion exchange resins in the preparation of fibrinogen and of globulins for clinical use.

Electrophoretic studies.—Moore *et al.* (160) have examined the effects on electrophoretic patterns of the storage and shipping of serum or plasma samples, of hemolysis in the sample, and of dialysis in phosphate-saline and in veronal buffers. Perlmann & Kaufman (161) draw attention to significant changes in pattern resulting from exposing serum to acetic acid below pH 4. The effect seems to be confined to the lipoproteins. Jager, Smith & Nickerson

(162) have carried out an electrophoretic analysis of plasma fractions II-1,2 and II-3. Both fractions behave as homogeneous antigens toward rabbit sera although physical methods demonstrate molecular heterogeneity; indeed, the two fractions appear to be immunologically equivalent although they differ in physical properties. The authors state that antisera to human γ-globulin react to a globulin in serum other than the γ-globulins with the result that immunological assays of γ-globulin are higher than are estimations based on electrophoretic analysis.

Alberty (163) has, also, made a careful study of the γ-globulins over a wide range of pH and of ionic strength. The isoelectric points vary considerably with ionic strength. The author indicates that although human and bovine albumin have the same isoelectric point their electrophoretic patterns in the isoelectric region differ significantly. Abrams & Cohen (164) have compared the electrophoretic behavior of fractions of the cytoplasmic proteins of human lymphoid and calf thymus tissue with that of the proteins of plasma. Electrophoretic comparison of the sera of 28 animals of low phylogenetic order have been made by Deutsch & McShan (165). Attention may appropriately be drawn to an ultracentrifugal analysis of fractions of porcine blood plasma by Koenig (166).

Plasma analysis: clinical methods.—Several papers have appeared on the determination of the A/G ratio by salting out. Derrien (167) recommends a phosphate mixture (pH 6.5) while Isbister (168) finds that 22.2 per cent of sodium sulfate (Howe's method) and 21 per cent sulfite give essentially the same results. Harkness & Whittington (169, 170) from a survey of the results of many analyses by Howe's method, come to the conclusion that there exists in plasma a complex of the albumin with the globulins and this complex is in equilibrium with an excess of free albumin. They assign a molecular weight of 104,000 to the complex and set up an equation containing a constant whose variations they believe to be of greater significance than is the A/G ratio.

Adaptations of the biuret reaction to the estimation of protein continue to appear (171, 172, 173), as do attempts to bring other color reactions, such as the Sakaguchi (174) and the xanthoproteic (175) reactions to the service of the clinical chemist. Goiffon (176) describes a turbidimetric method for serum globulin based upon the formation of tubidity when the proper amount of boric acid is added to serum.

The Reactions of Proteins

Anion binding.—The reversible combination of proteins with small molecules, particularly organic anions, has now developed into a major field of interest to protein chemists. A better understanding of the forces involved and of the curious specificities which are displayed may be expected to advance our knowledge of the fine structure of protein molecules and thereby to contribute to many problems of enzyme action, of immune specificity and of other physiological processes.

It is well known that the binding of some ions is highly specific with respect to the protein involved. The metallo-enzymes (177) and other metal-

lo-proteins are good examples. A striking case of specific binding of phosphate has recently been reported by Velick (131). Observing that the basic proteins aldolase and glycericaldehydephosphate dehydrogenase have isoelectric points below pH 7 in phosphate buffers, Velick investigated the binding of phosphate ions by aldolase. Equilibrium dialysis and electrophoretic measurements were consistent with a binding constant as high as 1,000—a value, incidentally, which is comparable with that of the Michaelis-Menten constant for aldolase and fructose diphosphate. Another interesting example is the formation of soluble complexes of the homologous hapten ion with the purified antibody to sheep serum diazotized with *p*-arsanilic acid. Eisen & Karush (178) find that only two centers in the protein appear to be involved in binding the hapten and that the binding constant has the high value of 3.5×10^5. Other pertinent work in the field of immune specificity cannot be reviewed adequately in this chapter but attention should be drawn to recent publications from Pauling's laboratory (179, 180) on the competitive inhibition of antigen-antibody interaction by simple molecules structurally related to the homologous hapten. This continuing study provides abundant evidence of the restricted configurational adaptibility of the hapten-binding sites of antibodies.

Apart from such specific reactions there is a general feeling that anion binding is a characteristic of proteins in general. Although this may be true it is becoming clear that proteins differ very much in their individual affinities for a particular ion. Klotz & Urquhart (181) have compared the extent of combination of methyl orange with ten crystalline proteins and three plasma globulin fractions. Only three of these, serum albumin, β-lactoglobulin and hemocyanin (182) combine significantly with the dye over the concentration range examined. The relative affinities may be represented by the ratio 10:3:1. The authors have sought an explanation for this specificity in some peculiarity in the amino acid composition of the active proteins. They suggest that, although all of the cationic groups in proteins may be potential binding sites for anions, their affinities may be modified profoundly by intramolecular competition of the carboxylic anionic groups of the protein and that the competitive affinity of the latter, in turn, may be opposed by hydrogen bonding with hydroxyl groups in the protein.

That the sites at which anions combine are the cationic groups in the protein is entirely plausible and is qualitatively supported (181, 183) by the observations that combination by serum albumin diminishes sharply as the pH rises above 10 and that it is diminished also by acetylation of the protein but not by conversion of amino to guanidine groups. On the other hand, evidence accumulates (184) that the free energies of binding of successive anions by a single protein diminish rapidly. It is this spread in affinities of reaction which complicates the problem of establishing, in a particular case, the maximum number of anions which can be bound, and correlating this with the structure of the protein. The simple application of the principle of mass action has not proved of general validity, even when allowance is made for the electrostatic effect of the bound ions (184). Lacking a dependable

method of formulating data, extrapolations to the maximum binding capacity are precarious and unreliable (184 to 187). It would appear that we need an extended study of a single protein with several types of anions over a very wide range of concentration such that measurements of low degrees of binding by equilibrium dialysis overlap observations of more extensive binding in which measurements of mobility, osmotic pressure, and acid-base equilibria are made and other appropriate methods are employed. In this connection, attention may be drawn to the description by Stricks & Kolthoff (188) of a polarographic method of studying the combination of methyl orange with protein.

Using arbitrary adaptations of the mass law, thermodynamic analyses of ion binding have been made in several cases (184, 185, 189). The results are consistent in indicating that the free energy of binding is largely an entropy effect. It would be plausible to attribute this to configurational changes in the protein. Klotz & Urquhart (185) have, however, advanced the suggestion that it may be due to the release of bound water as the result of the electrostatic bond between the protein and the anion.

Evidence continues to accumulate that the affinity of a single protein for anions varies widely with the nature of the ion. A problem of practical importance is the extent of binding of buffer anions at concentrations of buffers commonly employed in physico-chemical and biochemical studies of protein solutions. Klotz & Urquhart (190) find the following order of diminishing ability of simple anions to compete with methyl orange for serum albumin: phthalate, nitrate, citrate, veronal, chloride, phosphate, acetate. Glycine did not show any evidence of binding. Scatchard & Black (191) and Longsworth & Jacobsen (192) discuss theoretical aspects of the effects of binding on isoelectric points and present data on serum albumin and on β-lactoglobulin. From their observations they give the following series in increasing order of effectiveness: acetate, chloride, bromide, nitrate, iodide, thiocyanate, *p*-toluene sulfonate and trichloroacetate. Volkin (193) interprets the effect of 0.15 M thiocyanate on the mobility of insulin in the isoelectric region as consistent with the binding of nine to ten thiocyanate ions per mole of protein.

Denaturation.—The binding of ions by a protein may be accompanied by rather paradoxical effects on the native state of the protein. The anionic detergents, salicylates etc. are familiar denaturing agents at moderate concentrations and, at the same time, are good solvents for insoluble proteins. Under other conditions they appear to protect proteins from denaturation. For example, caprylate (194), aromatic carboxylates (181, 186) and cyanate (195) have been shown to prevent coagulation of serum albumin and egg albumin by heat and to prevent, also, the rise in viscosity which normally follows solution of these proteins in concentrated solutions of urea. In the latter case the question of competition for combination with the protein is evidently involved since the solvent effect of urea is associated with binding of urea molecules. Bresler (196) calculates from sedimentation data that one mole of serum albumin can combine with about 360 moles of urea. When gluten is dissolved by salicylate (197), zein by anionic detergents (198), or

isoelectric insulin by thiocyanate (193) there is good evidence of extensive binding of ions in each case. Physical measurements on the solution of zein were consistent with the conclusion that the protein was molecularly dispersed with little change in shape. In other cases there is good evidence of profound changes in molecular configuration (199). Pereira & Gonçalves (194) find that 0.1 *M* caprylate will protect egg albumin from heat denaturation but will facilitate its denaturation by 0.5 *M* salicylate. The products of denaturation of methemoglobin by 4.5 *M* urethane and by 0.5 *M* salicylate are indistinguishable spectroscopically, according to Schlegel & Johnson (200). Yet the effect of salicylate is reversed on dilution and is not temperature-dependent, whereas the reaction with urethane is sensitive to temperature and apparently irreversible.

The question of the effect of changing the distribution of charge on the protein has been raised by Huggins & Jensen (201). They have observed that the anions of iodo-, bromo-, and chloroacetic acid inhibit the coagulation of serum and of egg albumin by heat, whereas the neutral molecules iodoacetamide and methyl iodoacetate actually promote coagulation. Assuming that all of these compounds react with the protein by alkylation of thiol and, possibly, amino groups, the authors suggest that the protective effects of the anions are due specifically to the electrostatic repulsions of the negative charges which accumulate on the protein as a result of reaction with the anionic reagents. An interesting observation is that all of the compounds mentioned, whether ionic or not, tend to give a translucent rather than the usual opaque clot.

Jacobsen & Christensen (202) find a negative temperature coefficient in the denaturation of β-lactoglobulin by urea. To explain this and the kinetic data they derive a reaction mechanism involving two consecutive reactions controlled by a temperature-dependent equilibrium. Denaturation of this protein (203) is accompanied by a large increase in levo-rotation. Boyd & Eberl (204) apply the theory of absolute reaction rates to the published data on the heat inactivation of tobacco mosaic virus and propose a chain mechanism. The heats and entropies of inactivation of four phages of *E. coli* have been measured by Foster, Johnson & Porter (205).

Quantum yields for the inactivation of swine pepsin (206) and of ficin (207) by ultraviolet light (2,537 Å) have been determined by McLaren and associates. They suggest that there may be a rough inverse proportion between quantum yields and the molecular weights of proteins.

An interesting speculative paper by Kahn (208) should not be overlooked. The author develops the hypothesis that the key reaction in the denaturation of a protein is the formation of diketopiperazine rings between adjacent peptide chains. This theory of piperazine "kinks" in the peptide configuration is extended to embrace muscle contraction, chemiluminescence and protein synthesis.

Phosphorylation.—The phosphorylation of proteins has been further examined by Ferrel *et al.* (209) and by Boursnell *et al.* (210). Most of the phosphate groups are loosely bound and are lost on prolonged dialysis. Those

which are firmly bound are present as ortho or metaphosphate esters on aliphatic hydroxy and, possibly, on phenolic groups. The interesting point is made (209) that some peptides linkages seem to be more sensitive to hydrolysis in proteins in which the loosely bound phosphate is present.

Guanidation.—Details have now been published (211) of the use of O-methyl isourea to convert the amino groups of proteins into guanidine groups. Derivatives of serum albumin in which the numbers of the latter groups vary from 10 to more than 50 have been prepared. A crystalline derivative with 54 such groups is described.

Formaldehyde cross-linkage reactions.—Middlebrook (212) finds, in nine proteins, that the amounts of stably bound formaldehyde correspond closely with the amounts of aspartic acid present. Accepting the view that this type of bound formaldehyde is combined in cross-linkage with amide groups, the author draws the conclusion that all of the aspartic acid in these proteins is present as asparagine residues.

Miscellaneous Physico-Chemical Studies

Electrophoresis.—Interest continues to be shown in analysis of the patterns of mixtures of proteins in which the components are incompletely resolved. Cann (87) has examined the overlapping boundaries of crystalline ovalbumin and the variation in the apparent concentrations of the two main components as the concentration of protein is changed. He finds the theoretical treatment of Dole (213) to be adequate for the description of his observations and derives the "true" concentrations by extrapolation to zero protein concentration. Hoch, also, has emphasized the importance of the total concentration of protein, particularly (214, 215) in the analysis of serum patterns. His most recent contribution (216) outlines a new method of measuring the relative mobilities of two components in an incompletely resolvable mixture. A solution of one component, in concentration equal to that in the mixture, is placed on top of the mixture. The migrations of the two boundaries are then followed simultaneously. It is claimed that, under limiting conditions, the change in the distance between the two boundaries is a measure of the difference in mobilities of the two components. The new procedure is offered as a supplement to the usual method of measurement of individual boundary displacements in two separate experiments.

Anderson & Alberty (217) analyze reversible boundary spreading and steady state criteria in relation to the question of electrophoretic homogeneity. None of the 14 crystalline proteins which the authors studied fulfill their criteria of homogeneity. The theoretical effects of ionic strength and of ion binding on isolectric points are discussed by Scatchard & Black (191) and by Longsworth & Jacobsen (192).

Sedimentation.—Goffman, Lindgren & Elliott (218) come to the conclusion that the boundary asymmetry of albumin in undiluted serum is due to what they describe as a "piling up" of lipoprotein on the albumin concentration gradient. The usual methods of analyzing these boundaries lead to large errors in the amounts of albumin and lipoprotein. The analysis of the

authors gives amounts of lipoprotein which are much more closely in agreement with electrophoretic estimates of the β_1-lipoprotein than are hitherto published estimates based on sedimentation patterns.

Gutfreund & Ogston (219) have described a method of obtaining the sedimentation constants of molecules too small to form a clear sedimentation boundary in the ultracentrifuge. The principle involved is the measurement of the rate at which material moves across an arbitrary plane in the cell. Preliminary results are reported on the application of the method to the partial degradation products of oxidized insulin.

Gelatin.—Solutions of ichthyocol (from the swim-bladder of the carp) degrade rapidly at pH 2.5 (220). An analysis of the accompanying changes in viscosity leads to the inference that there is a parent gelatin molecule with a length of 800 Å and an axial ratio of 47.5. The rigidities of the gels of a series of gelatins of graded average molecular weight have been studied by Ferry (221, 222) and the changes in optical rotation which accompany gelling have been examined. Joly (223, 224) has continued his investigation of the effects of various organic substances on the birefringence of flow and the gelling properties of gelatin. Barbu & Macheboeuf (225) suggest that the curve relating the temperature of gel formation to pH is an individual characteristic of a protein.

Surface reactions.—Studies are continuing in Bull's laboratory (226) of the behavior of protein monolayers at pressures below 1 dyne per sq. cm. The areas of extended films of pepsin depend on the spreading concentration and on the time elapsing between spreading and compression. It is thought that a duplex film is first formed and that this changes quickly to a monolayer which then expands slowly to something corresponding to a β-keratin film lying flat on the surface. There is evidence of the dissociation of insulin in the surface at low pressures. Ellis & Pankhurst (227) prepare stable films of gelatin by spreading on 30 per cent ammonium sulfate from formic acid solution. Two critical areas of 20 Å and of 27.5 Å respectively are found. Below a certain critical pressure the viscosity of a protein monolayer (228) is found to be independent of the velocity gradient. When calculations of the viscosity coefficients were made, from accepted molecular dimensions of the proteins examined, very high values were obtained. The author concludes that the unit of flow in the film at limiting velocity gradients must be smaller than a molecule. Indeed, he calculates that this unit was the same in all the proteins studied and corresponded with an area of 90 sq. Å.

Infrared analysis.—Goldstein & Halford (229) and Ambrose, Elliott & Temple (230) have examined various preparations of collagen, keratin, and myosin by polarized infrared light. By varying the orientation of the specimens relative to the angle of incidence and the plane of polarization, information is sought on the fine structure of the specimens.

The presence of amide carbonyl groups in a film of dried serum albumin is clearly demonstrable in infrared spectra (231, 232). Since the dried albumin still retained its native ability to bind anions, Klotz & Griswold (231) con-

clude that the amide groups are not a product of denaturation but are present in the native molecule.

Electron microscopy.—Schmitt (233) and Wyckoff (234) have published reviews of the application of the electron microscope to the elucidation of the fine structure of macromolecules and of biological microstructures. Hall (235) has deduced the unit cell of edestin. The molecule is approximately spherical, having a diameter of 80 Å and a computed molecular weight of 290,000. The same investigator (236) has found, in specimens of human fibrinogen, that the protein is arranged in fine filamentous strings of beads of diameter 40 Å and lengths varying from 300 to 1,100 Å. Fibrin preparations were also examined. It is suggested that fibrin is formed by lateral aggregation of fibrinogen filaments. Wolpers (237) identifies a period of 640 Å in collagen preparations and distinguishes dense and transparent parts within this period.

X-ray diffraction.—Astbury (238) analyzes the diffraction patterns of polyglycine and Hughes & Moore (239) have worked out the molecular configuration in the β-crystals of glycylglycine. The molecule is coplanar except for the NH_3^+ groups. The interatomic distances are all normal. The short carbon to nitrogen distance, which has previously been reported in the patterns of amino acids, is not found in glycylglycine.

Perutz (240) has calculated a complete three-dimensional Patterson synthesis for horse methemoglobin. The molecule resembles a cylinder having a diameter of 57 Å and a height of 34 Å in which the polypeptide chains lie parallel to the base. The chains are arranged in four layers in which the flat sides of the heme groups are normal to the chain direction. Perutz offers the suggestion that crystals of globular proteins may simply be assemblies of parallel chains in α-keratin configuration.

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NUCLEIC ACIDS, PURINES, AND PYRIMIDINES[1]

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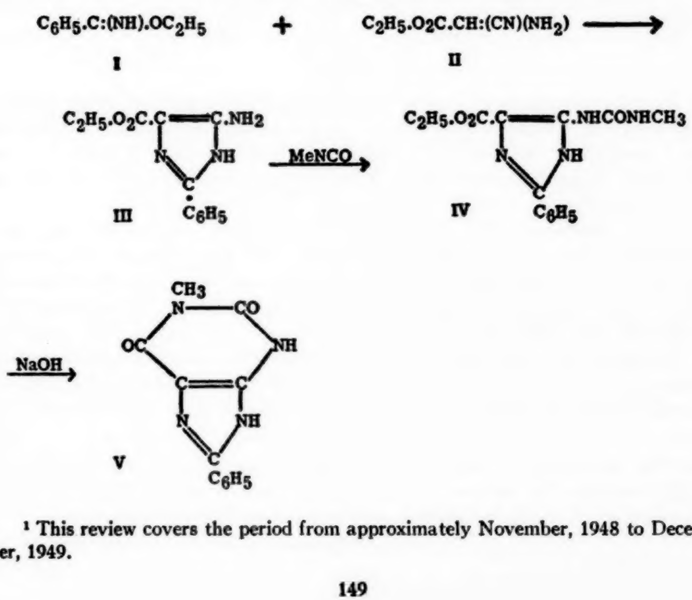
Synthetic chemistry of pyrimidines and purines.—Studies of the synthetic chemistry of nucleic acids and related compounds have been intensely pursued from several viewpoints. Apart from the obvious significance of synthetic methods for the elucidation of structural problems, such studies have been stimulated by the necessity of developing suitable procedures for the preparation of labelled purines and pyrimidines and of related compounds concerned in nucleic acid metabolism. An excellent review on recent aspects of the chemistry of pyrimidines and purines has been given by Lythgoe (2).

A new sequence of reactions leading from α-aminonitriles (amino cyanoacetates) through thiazole or imidazole derivatives to the synthesis of purine compounds was described by Cook *et al.* (3, 4), Heilbron (5), Downer *et al.* (287), MacDonald and co-workers (288). For example, benzimino ethyl ether (I) was condensed with the ethyl ester of amino cyanoacetic acid (II) to 4-amino-5-carbethoxy-2-phenylglyoxaline (III). This compound, on refluxing with a large excess of methyl isocyanate, yielded 4-N′-methyl ureido-5-carbethoxy-2-phenylglyoxaline (IV). On treatment with alkali and subsequently with acid, 8-phenyl-1-methylxanthine (V) was obtained.



[1] This review covers the period from approximately November, 1948 to December, 1949.

This synthesis is of particular interest because of the close similarity of the intermediary imidazole derivative to a compound which was isolated from *E. coli* cultures during sulfonamide bacteriostasis, according to Stetten & Fox (6), and which was identified by Shive *et al.* (7) as 4-amino-5-imidazole carboxamide. It is probably an intermediary of the biological synthesis of purines by this microorganism. A new laboratory synthesis of this compound has recently been reported by Shaw & Woolley (8).

Preparation of labelled natural purines and pyrimidines.—The preparation of 1,3-N^{15}-labelled uric acid as well as that of uric acid containing the N^{15} probably in the 9 position was described by Cavalieri *et al.* (9). In the first synthesis, 1,3-N^{15}-aminobarbituric acid (uramil) was condensed with potassium cyanate to 1,3-N^{15}-pseudouric acid; in the second, unlabelled uramil was condensed with labelled urea with loss of one of the labelled NH_2 groups to 9-N^{15}-uric acid.

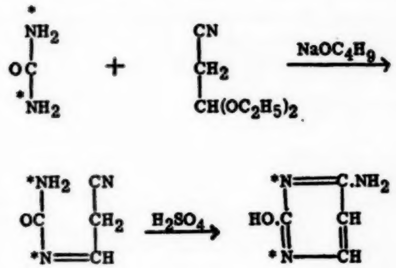
Bendich *et al.* (10) and Cavalieri *et al.* (11) developed an improved procedure for the preparation of 1,3-N^{15}-labelled adenine which is based on the method of Badilley, Lythgoe & Todd (12). The isotopic nitrogen is introduced during the synthesis of the intermediary formamidine from ethyl-formimino ether and N^{15}-ammonia. The atom per cent excess of N^{15} is equally distributed between both nitrogen atoms.

The same authors (11) obtained 4,6-C^{13}-labelled adenine from labelled malononitrile. Cavalieri & Brown (13) synthesized 8-C^{13}-labelled adenine by condensation of 4,5,6-triaminopyrimidine with C^{13}-formic acid to 4,6-diamino-5-formamidopyrimidine. Ring closure was effected by heating in formamide.

The preparation of 1,3-N^{15}-labelled guanine from guanidine has been described earlier by Plentl & Schoenheimer (14). In this paper the preparation of 1,3-N^{15}-uracil and 1,3-N^{15}-thymine has also been reported; the pyrimidines[2] were obtained from labelled cyanamide by way of thiourea according to Wheeler & Liddle (15).

Bendich *et al.* (16) have synthesized 1,3-N^{15}-labelled cytosine (16) by refluxing labelled urea with cyanoacetaldehyde diethylacetal in the presence of sodium butoxide and by cyclisation by treatment with dilute sulfuric acid.

[2] The numeration of the positions in the pyrimidines is not consistent in the current literature. In this review the designations used in the corresponding papers are employed. It would be desirable to arrive at a uniform nomenclature.

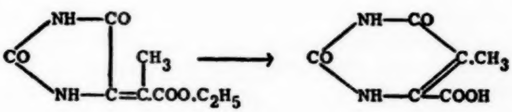


A synthesis of cytosine and methyl cytosine which might be helpful in the preparation of labelled compounds has been developed by Hitchings *et al.* (17). It is based on the observation (18) that the 4-thiol group of 2,4-dithiolpyrimidines is more reactive toward ammonia than the 2-thiol group.

Mentzer & Billet (19) reported a new synthesis of thymine-4-carbonic acid (methyl orotic acid) by condensation of urea with the ethyl ester of oxalopropionate (VI) in acetic acid in a current of dry hydrochloric acid. Carbethoxy-ethylidene-hydantoin (VII) is formed, which yields thymine-4-carbonic acid (VIII) on treatment with alkali.

$$CH_3.CH(COOC_2H_5).CO.COO.C_2H_5 + CO(NH_2)_2 \longrightarrow$$

VI

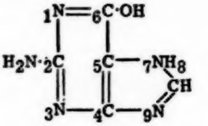


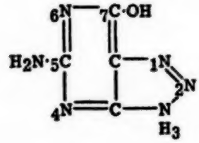
VII VIII

Mitchell & Nyc (20) had earlier observed a corresponding hydantoin formation when they condensed urea with oxaloacetate.

Bendich *et al.* (10) have also synthesized 1,3-N^{15}-isoguanine (2-hydroxy-adenine) by condensation of labelled thiourea with malononitrile to 4,6-diamino-2-thiolpyrimidine, desulfuration, introduction of a nitroso group in the 5-position followed by subsequent reduction to 4,5,6-triamino-2-hydroxypyrimidine, and finally by ring closure of the formamido compound after heating with a mixture of formic acid and formamide. (Reductive desulfuration of thioisoguanine obtained from 4,6-diamino-2-thiolpyrimidine in analogous procedures yielded adenine.) 2,6-N^{15}-diamino purine was obtained by Bendich *et al.* (10) by treating 2,4,5,6-tetramino pyrimidine (21) with a mixture of formamide and formic acid.

Triazolo analogues of the natural purines adenine, hypoxanthine, guanine, and xanthine have been synthesized by Roblin *et al.* (22) by diazotizing the corresponding aminopyrimidines.





Guanine
(2-amino-6-hydroxypurine)

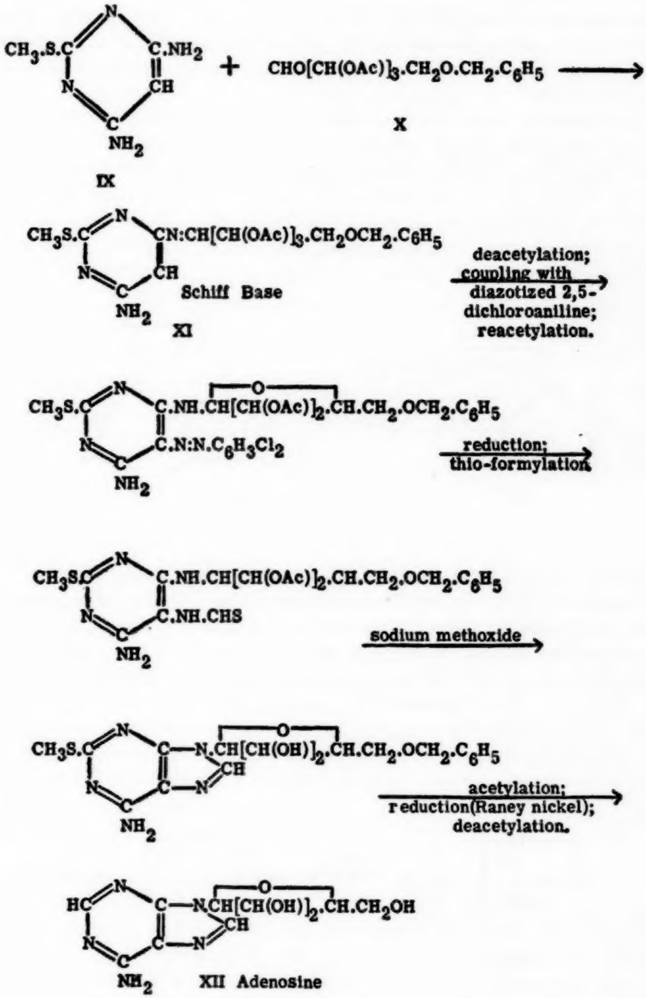
Triazologuanine
(5-amino-7-hydroxy-1-v-triazolo[d]-pyrimidine)

Synthetic chemistry of nucleosides and nucleotides.—During the development of routes for the synthesis of natural and model nucleosides in Todd's laboratory,[3] it was found that some synthetic procedures resulted in the formation of ribonucleosides which differed from the corresponding natural nucleosides in the ring structure of the carbohydrate component. Whereas all known natural ribonucleosides are β-furanosides, the ribonucleosides obtained by synthesis were frequently β-pyranosides. Recently, the authors were successful in establishing routes which prevented the formation of pyranosides and led to the synthetic preparation of β-furanosido nucleosides.

A brief outline of two important pathways illustrates some of the results of these investigations: (*a*) the "Schiff base route" for the synthesis of purine nucleosides [e.g., adenosine, (25)] is based on the condensation of a suitable 4,6-diaminopyrimidine derivative (e.g., 4,6-diamino-2-methylthiopyrimidine, IX) with 2,3,4-triacetyl-5-benzylribose (X) with the formation of the corresponding Schiff base (XI). After deacetylation of the carbohydrate groups, the Schiff base isomerizes to a D-5-benzylribofuranoside. An amino group is then introduced into the 5-position of the pyrimidine ring by coupling with diazotized 2,5-dichloroaniline. After reacetylation, thioformylation of the 5-amino group, and ring closure in the presence of sodium methoxide, the nucleoside (XII) is obtained by reductive debenzylation, followed by deacetylation.

The synthesis of β-9-ribofuranosidoadenosine (XII) has recently been accomplished by the route outlined (25). The important innovation introduced by Todd and his associates into their original technique of the "Schiff base route" consists in the use of the benzyl group for the protection of the 5′-position of the ribose derivative. The modification prevented the forma-

[3] For reasons of space, only the synthesis of naturally occurring ribonucleosides has been considered in the text. The preparation of other nucleosides is obviously of great interest for studies in the field of enzyme specificity and of metabolic antagonism. The recent syntheses of the arabofuranosides of theophylline and of adenine (23) and of 9-D-galactosido-2-methyl thioadenine (24) have been undertaken with this aspect in Todd's laboratory.

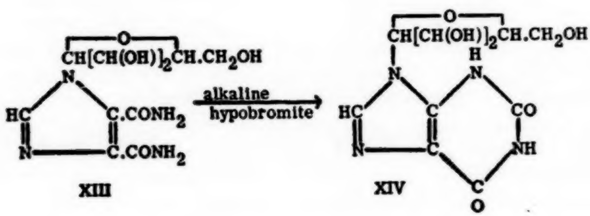


tion of pyranosides which occurred frequently when other protective groups such as benzoyl had been employed instead.

(*b*) Condensation of acetobromo-D-ribofuranose with the silver salt of a suitable base. This route was made possible by the synthesis of 1,2,3,5-tetraacetyl-D-ribofuranose (26) from 1,2,3-tetraacetyl-5-trityl-D-ribofuranose (27) and its subsequent transformation to acetobromoribofuranose.

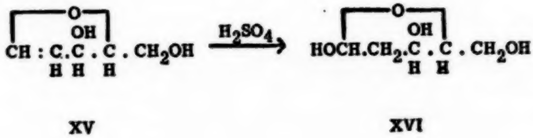
An example of this pathway (28) is the synthesis of adenosine by condensation of the silver compound of 2,8-dichloroadenine with acetochlororibofuranose, deacetylation and hydrogenation with palladised barium sulfate as catalyst.

Synthesis of xanthosine.—Condensation of acetobromo-D-ribofuranose with the silver salt of glyoxaline-4,5-dicarboxylate followed by treatment with ammonia yielded 1-D-ribofuranosidoglyoxaline-4,5-dicarboxamide (XIII). Treatment of the amide with hypobromite (Hofmann reaction) yielded 9-β-furanosidoxanthosine (XIV) (29). The 9-β-furanosido nucleosides of adenine, guanine and cytosine had been synthesized earlier with the help of acetobromo-D-ribofuranose by Todd *et al.* [For references see (29)].

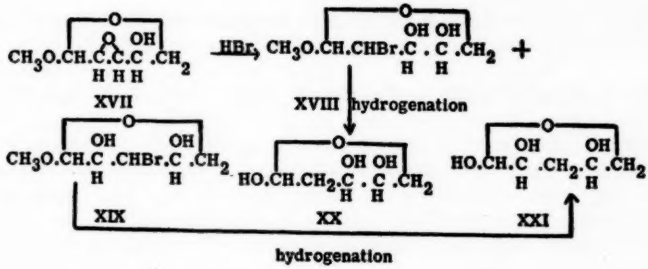


Several nucleosides with methylated adenine and guanine groups have been obtained by Bredereck, Haas & Martini (30), who used dimethylsulfate as the methylating agent of adenosine and guanosine. The influence of the methylated nucleosides on diuresis and blood pressure was studied.

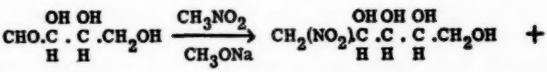
Synthesis in the field of desoxy sugars.—Synthetic investigations in the field of desoxy sugars have recently been conducted in the laboratories of Stacey and of Todd. Three routes for the synthesis of D-desoxyribose have been described, for which the yields so far obtained have been low: (*a*) The hydration of arabinal (XV) to desoxyribose (XVI) by acids [Meisenheimer



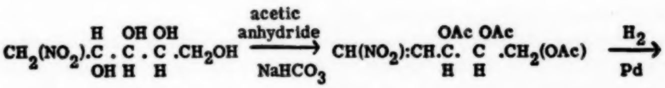
& Jung (31)] was improved by Deriaz *et al.* (32). (*b*) D-Desoxyribose has been prepared from 2,3-anhydro-β-methyl-D-riboside (XVII) (33) which, on treatment with hydrogen bromide, yielded the expected mixture of 3-bromo-β-methyl-L-D-xyloside (XIX) and 2-bromo-β-methyl-D-arabinoside (XVIII). The bromomethylpentosides can be separated and hydrogenated to 3-desoxyxylose (XXI) and 2-desoxyribose (XX) respectively. [See also (34)]. (*c*) The aldehyde group of D-erythrose can be condensed with



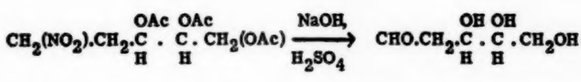
nitromethane (35) in the presence of sodium methoxide in analogy to the procedure used by Sowden & Fischer (36, 37). On acetylation and subsequent treatment with bicarbonates, the corresponding acetylated 1-nitro-1,2-olefine is obtained. This reaction had been applied earlier by Schmidt & Rutz (38) to 1-nitro-2-acetoxy paraffins. Reduction of the olefine group followed by deacetylation and conversion of the nitro to the aldehyde group [Nef (39), Sowden & Fischer (37)], by treatment with acid yields the 2-desoxy-D-ribose which was isolated as the anilide (35).



D-Erythrose D-ribonitropentitol



D-arabonitropentitol 1-nitro-D-erythro-3,4,5,-triacetoxypent-1-ene



Another procedure for the transformation of certain derivatives of anhydro sugars to the corresponding desoxy sugar compounds is hydrogenation with lithium aluminum hydride (40, 41). Prins (42) succeeded in obtaining by this method 2-desoxy-4,6-benzylidene-α-D-allopyranoside from 2,3-anhydro-4,6-benzylidene-α-D-allopyranoside (43). Syntheses of 2-desoxyglucosides of pyrimidines by way of 2-bromo-triacetylglucosides have been reported by Goodman & Howard (44).

Synthetic methods of phosphorylation.—A new and highly important reaction between neutral, benzylated esters of phosphorus, phosphoric and pyrophosphoric acids, and tertiary bases has been discovered by Baddiley, Clark, Michalski & Todd (45) This reaction leads to the selective removal

of one of the benzyl groups from such esters according to the following scheme:

$$R_3N + P_2O_3[OCH_2.C_6H_5]_4 = [R_3N.CH_2.C_6H_5]^+ + {}^-OP_2O_3.[OCH_2.C_6H_5]_3$$

Preparation of nucleosides and nucleotides from nucleic acid hydrolysates.—The older procedures for the isolation of the pyrimidine nucleotides and nucleosides from hydrolysates of ribonucleic acids have been replaced by more expedient methods. Ribocytidine (yield 3 per cent of the nucleic acid) and ribouridine (yield 12 per cent) were obtained by Loring & Ploeser (46) by refluxing yeast ribonucleic acid with 0.4 *N* sulfuric acid for 14 hr. and for 46 hr. respectively. The cytidine nitrate obtained was converted to the free base with the help of ion exchangers. Although the yield of cytidine is not as high as that obtained by Bredereck, Martini & Richter (47), the procedure of Loring & Ploeser seems to be less time-consuming than the method of these authors when the preparation of the purine nucleosides can be omitted. The preparation of uridine according to Loring & Ploeser is in part based on the deamination of cytidine during prolonged acid hydolysis and permits thus the isolation of the base in high yield.

An improved procedure for the preparation of cytidylic and uridylic acids from ribonucleic acid hydrolysates has been described by Barker, Gulland, Smith & Thomas (48); the separation of the two pyrimidine nucleotides is accomplished by the precipitation of cytidylic acid with pyridine in which uridylic acid is soluble [Bredereck & Richter (49)]. An alternative procedure is that of Loring, Roll & Pierce, who fractionated the pyrimidine nucleotides with 12-phosphotungstic acid (50).

Preparation of desoxyribonucleosides.—A new method for the chromatographic fractionation of the mixture of desoxyribonucleosides obtained by the enzymatic dephosphorylation of desoxyribonucleate according to Thannhauser *et al.* (51, 52) was described by Schindler (53). Crude desoxyguanosine settled out after concentration of the digest, desoxyhypoxanthosine crystallized from the mother liquors of desoxyguanosine after addition of alcohol, thymidine and desoxyxanthosine were prepared from the remaining mother liquors by chromatographic adsorption on aluminum oxide.

Preparation of nucleic acids.—Signer *et al.* (54) introduced some modifications into Hammarsten's technique for the preparation of fibrous desoxyribonucleic acid (DNA), with the purpose of preventing enzymatic depolymerization during the extraction of the fresh glands. According to Dounce (55), however, it is doubtful if any of the extraction procedures devised for the preparation of fibrous desoxyribonucleates permit the isolation of DNA in unaltered form. He found that the DNA of nuclei which were isolated at approximately neutral pH form gels on addition of ammonia or salt solutions of high ionic strength, and that it is very difficult to extract desoxyribonucleate from such gel forming nuclei. On the other hand, nuclei isolated at pH 6 do not form gels and permit the extraction of DNA.

The preparation of highly polymerized DNA from yeast and from avian

tubercle bacilli has been described by Chargaff *et al.* (56, 57). Kerr & Seraidarian (58) described a procedure for the preparation of pancreas ribonucleic acid (RNA).

Preparation of labelled nucleic acids and nucleic acid derivatives.—The preparation of N^{15}-labelled RNA from yeast cultured in an N^{15}-containing medium has been described by Hammarsten (59) and by Di Carlo, Schultz, Roll & Brown (60), who found that the N^{15} was equally distributed between guanine, adenine and the pyrimidine groups. Hammarsten, Reichard & Saluste (61) obtained N^{15}-labelled cytidine and uridine from the N^{15} labelled RNA prepared in this way in an N^{15}-containing medium. The isolation of purines and pyrimidines by chromatographic methods from small amounts (milligrams) of RNA and DNA has been described by Reichard (62) and by Edman, Hammarsten, Löw & Reichard (63).

Preparation of desoxyribonucleoproteins.—The isolation of nucleoproteins, in particular the technique of their extraction from tissues, has become a peculiar problem ever since Bang (64) and Hammarsten (65) demonstrated the dissociating effect of high sodium chloride concentrations on thymonucleohistone. Most of the recent procedures make use of Mirsky & Pollister's (69) observation that 1 *M* salt solutions are solvents for some desoxyribonucleoproteins. Thomas & Mayer found that the nucleoproteins of mammalian sperm cannot be extracted by 1.0 or 2.0 *M* sodium chloride (66). It is no easy task to decide whether or not the desoxyribonucleoproteins obtained by extraction with 1 *M* salt solutions represent the genuine substances. There is evidence that at such salt concentrations a partial dissociation into nucleic acids and proteins already takes place and that the physicochemical properties of such preparations are in part due to the presence of free nucleate and free histone. In view of the colloidal and polyvalent nature of both components, it is unlikely that a reversion of this process would lead to the recombination to the natural nucleoproteins. Stern *et al.* (67) reported that thymus nucleohistones extracted with solutions of low ionic strength have a much lower degree of asymmetry than those extracted with 1 *M* salt solutions. These difficulties of interpreting the physicochemical properties of nucleoproteins obtained by extraction with 1 *M* salt solutions do not affect the value of these procedures for the investigation of certain chemical properties of nucleoproteins.

A procedure for the preparation of desoxyribonucleohistone from spleen (and thymus) has been described by Peterman & Lamb (68). In this work, the action of desoxyribonuclease, which is present in spleen in considerable amounts, was inhibited (*a*) by maintenance of low temperature throughout the preparation, (*b*) by the presence of citrate which removes the activating magnesium ions, and (*c*) by extraction with 1 *M* salt solutions. The nucleohistone obtained by Peterman & Lamb forms solutions of higher viscosity than similar preparations from other sources obtained earlier by Mirsky & Pollister (69) and by Stern *et al.* (67), who used arsenate ions for the inhibition of desoxyribonuclease during the extraction at low ionic strength.

Frick (70) investigated some physicochemical properties of desoxyribo-

nucleohistone prepared from calf thymus according to Mirsky & Pollister (71) by extraction with 1 *M* sodium chloride and precipitation by dilution. The histone gave a negative tryptophane test. In the ultracentrifuge, the nucleohistone solutions had polydisperse properties; the presence of three components was demonstrated by electrophoresis. The nucleoprotein solution in 1 *M* sodium chloride is highly viscous. In contrast to the behavior of desoxyribonucleate solutions (72) the addition of guanidinium salts does not cause a decrease of the viscosity of the nucleoprotein solutions. On fractional precipitation by dilution with water, the ratio of nucleic acid to protein in the precipitates remains practically constant.

V. Euler & Heller (73) described the preparation of cytoplasmic ribonucleoprotein fractions from liver homogenates. After the removal of nuclei and mitochondria, the liponucleoproteins of the supernatants were precipitated with acetic acid or calcium chloride or streptomycin. The precipitates obtained with the last two reagents could be redissolved in salt solutions and reprecipitated by dialysis. The RNA content of the precipitates obtained with calcium chloride or streptomycin was considerably higher than that of the precipitates obtained with acetic acid. The nucleoproteins of birch pollen have been studied by v. Euler *et al.* (74).

Hultin & Herne (75) compared the amino acid contents of basic sperm proteins of sea urchins and molluscs. The minimum molecular weights of these proteins are considerably higher than those of protamines. The basic sperm proteins of echinoderms are characterized by high contents of lysine, those of molluscs by high contents of arginine.

Determination of Individual Hydrolysis Products of Nucleic Acids

Analytical chemistry of purines, pyrimidines, nucleosides and mononucleotides.—A particularly important contribution of the last two years was the development of highly sensitive quantitative micromethods for the fractionation and determination of purines, pyrimidines, nucleosides and nucleotides.

Absorption spectra of purine and pyrimidine compounds.—Data concerning the light absorption of purine and pyrimidine compounds can be found in the following references: ultraviolet absorption of purines and pyrimidines (76), of purines, pyrimidines, and triazolopyrimidines (77), of pyrimidine ribonucleosides and ribonucleotides (78), of alloxan (79), of barbiturates (80); infrared absorption of nucleic acids, nucleotides and nucleosides (81), of substituted pyrimidines (82). In addition, almost all investigations on spectrophotometric methods (see below) in the nucleic acid field contain absorption data of pure compounds.

Counter current distribution.—Tinker & Brown (83) found counter current distribution to be an excellent method for the fractionation and characterization of small amounts (milligrams) of purines and pyrimidines.

Colorimetric Methods.—Glazko & Wolf (84) found that adenine, adenine nucleotides, and nucleic acids formed diazotable substances on reduction

with zinc and hydrochloric acid. On coupling with the Bratton-Marshall reagent (85), an orange color appeared with a maximum absorption at 505mμ. The optical density was proportional to the concentration of adenine. Guanine, cytosine, isocytosine, uracil, thymine, and thiamine do not produce colored derivatives under these conditions. A similar reaction is given by folic acid (86) which can, however, be distinguished from that of adenine derivatives by replacing zinc and hydrochloric acid with titanous chloride. Adenine derivatives produce no diazotable substances with this reducing reagent.

A colorimetric method for the determination of uracil and cytosine (6 to 33 γ) was described by Soodak, Pircio & Cerecedo (87). It is based on the fact that uracil and cytosine, after bromination, reduce a uric acid reagent. The quantitative separation of the two bases can be accomplished by an ion exchanger (Decalso).

Woodhouse (88) described a colorimetric method for the determination of thymine in nucleic acids. The procedure is based on Hunter's (89) color test for thymine requiring coupling with a diazo reagent and subsequent addition of a reducing agent (hydroxylamine). The application of the color reaction to bound thymine is possible only after liberation of the thymine by hydrolysis and its separation from purines and other interfering substances by fractional precipitation with silver reagents. The method, which requires about 50 mg. of DNA, permits the determination of bound thymine with a recovery of approximately 90 per cent.

Chromatographic methods.—The introduction of new techniques such as chromatography and microbiological assays has finally bridged a gap, in the available analytical tools, which has so far impeded nucleic acid research: the problem of the quantitative partition of the pyrimidine containing units. These techniques are now permitting the quantitative determination of the pyrimidine nucleosides and nucleotides without necessitating the quantitative liberation of the free bases—a requirement of the older pyrimidine determinations which could never be accomplished due to the great stability of the pyrimidine-carbohydrate linkages.

In the analysis of the purine-containing units, where satisfactory older methods are available, these techniques have greatly increased the sensitivity of the analytical methods. In addition, they have rendered possible discoveries of fundamental importance such as the chromatographic isolation of a new isomer of the adenylic acid series, presumably adenosine-2-phosphate, by Carter (90) and Cohn (91).

Vischer & Chargaff (92), who introduced the technique of paper chromatography into the analysis of the nitrogen-containing components of nucleic acids, described procedures for the separation and determination of purine and pyrimidine bases with butanol mixtures as solvents. The chromatograms can be developed either by fixing the bases on the paper strips by precipitation with mercuric salts and subsequent treatment with ammonium sulfide or by extraction of the bases with acid from segments of the paper strips and subsequent ultraviolet spectrophotometry.

In a later paper, Chargaff *et al.* (93) developed the partition of the nucleotides in ribonucleic acid hydrolysates by paper chromatography. The separation was carried out in an ammonia atmosphere with isobutyric acid as solvent. This procedure is an indispensable supplement to the earlier procedure, since it avoids the necessity of the liberation of the free pyrimidine bases which leads to errors by the secondary deamination of cytosine as well as by the incomplete liberation of uracil.

Hotchkiss (76) developed a procedure for the partition of purines, pyrimidines and some nucleosides by paper chromatography with butanol as solvent. His chromatograms of desoxyribonucleic acid hydrolysates show the presence of a small amount of an unidentified ultraviolet-absorbing component which might suggest the possibility that nucleic acids contain other than the known basic groups. Extensive data regarding the ultraviolet absorption of the purines and pyrimidines may be found in the paper.

Holiday & Johnson (94) localized the spots of purine and pyrimidine-containing substances by observation in ultraviolet light (230 to 400 mμ) against a background of paper fluorescence. Markham & Smith (95) used photography in ultraviolet light for the same purpose.

In the procedure of Smith (96) for the quantitative partition of purines, pyrimidines, nucleosides and nucleotides, the chromatogram is developed photographically by exposure to monochromatic light of 254 mμ.

The chromatographic separation of purines and pyrimidines by adsorption on a starch column with a mixture of propanol and 0.5 *N* HCl as solvent was described by Daly & Mirsky (97).

Microbiological methods.—Loring, Ordway & Pierce (98) developed a microbiological method for the determination of pyrimidine ribonucleosides, based on the growth curves of *Neurospora* mutant strain No. 1298 which requires pyrimidine ribonucleosides. The partition of the nucleosides into cytidine and uridine is possible when the microbiological assay is combined with the chemical separation by the selective precipitation of cytidine with 12-phosphotungstic acid.

A microbiological procedure for the identification of the guanine and adenine spots obtained by paper chromatography was described by Fries (99) and Fries & Björkman (100). The paper strips are sprayed with suspensions of conidia of appropriate *Ophiostoma* or *Neurospora* mutants and subsequently placed on agar-plates. Mycelia develop within 36 hr. around the spots containing the required bases. A similar procedure for bacterial growth factors was described by Winston & Eigen (101).

Hoff-Jørgensen (102, 103) found that the requirement of desoxyribosides for the growth of *Thermobacterium acidophilus R 26* can be used for the microbiological determination of the total desoxyribosides.

Determinations of pentoses and desoxypentoses.—The color reactions for both nucleic acid sugars have been studied in detail with the purpose of improving the criteria of their reliability for the quantitative determination of nucleic acids. McRary & Slattery (104), Brown (105) and Drury (106) reported dichromatic spectrophotometric procedures designed to eliminate

errors caused by the presence of other sugars, particularly of glucose, in the application of Bial's test for the quantitative determination of pentoses. Vasseur (107) studied spectrophotometrically the reactions of various sugars with 1 per cent orcinol and 20 volume per cent sulfuric acid. Characteristic curves for various carbohydrates were obtained, and conditions for the detection of individual sugars in mixtures and for their quantitative determination were established. A modified procedure for the determination of pentoses, nucleosides, and nucleotides was described by Barrenscheen & Vályi-Nagy (108).

Dische (109) developed a new method for the spectrophotometric determination of free and of bound pentoses which is based on the reactions of sugars with strong sulfuric acid followed by treatment with cysteine. Amongst the many applications of this group of reactions is the determination of ribonucleic acid in the presence of large amounts of desoxyribonucleic acid.

Deriaz, Stacey, Teece & Wiggins (110) studied the mechanism and the specificity of Dische's diphenylamine reaction. They confirmed the specificity of the characteristic blue color obtained with desoxyribose, and found that only ω-hydroxylevulinic aldehyde, arabinal, and furfuryl alcohol gave a similar reaction. A distinct absorption band at 5,800 Å is characteristic for the reaction product obtained with these substances. ω-hydroxylevulinic aldehyde, $CH_2OH \cdot CO \cdot CH_2 \cdot CH_2 \cdot CHO$, formed by the effect of acids on the precursors is most likely responsible for the formation of the pigment; no color was obtained with levulinic acid, one of the main end products of the reaction of desoxyribose with acids.

Li & Stacey (111) and Overend & Stacey (112) have reinvestigated the mechanism of the Feulgen nucleal reaction obtained with desoxyribonucleic acids. They confirmed earlier observations that, under carefully controlled hydrolysis conditions, the liberated reacting aldehyde groups remain in the acid insoluble fraction of the hydrolysis products—a prerequisite for the quantitative histochemical application of the nucleal reaction.

Determination of Nucleic Acids

Chemical procedures.—The analytical partition of the two main groups of nucleic acids (DNA and RNA) (after removal of acid soluble and of lipid P compounds) can be accomplished in two ways: (*a*) By extracting the total amounts of nucleic acid with hot trichloroacetic acid and by subsequent colorimetric determinations of ribose and desoxyribose in the extract [Schneider (113)]. Schneider's calculation of the RNA values from the ribose determinations is supported by the results of Belianski (114), who reported that only the purine nucleotides of ribonucleic acid produce color with the orcinol reagent, under the conditions of Mejbaum's procedure. (*b*) By hydrolysis with alkali at 37° [Schmidt & Thannhauser (115)]. Under these conditions RNA is transformed into acid soluble compounds, whereas DNA is quantitatively precipitated on acidification of the alkaline hydrolysate. The values obtained with either procedure in animal tissues agree satisfactorily.

A method incorporating both principles has been developed by Schneider

(116). It appears that the combined method offers certain advantages over either of the two different procedures. Since the publication of both procedures in 1946, a considerable amount of information concerning their applicability has become available from various laboratories. Whereas the general usefulness of both procedures appears to have been established, some of the recent experiences must be considered in the interpretation of the figures obtained. It should be pointed out that the original methods are valid as such only if applied to animal tissues. In plants and microorganisms, the possible interference of substances such as pentose polysaccharides, phytic acid, polyphosphates, requires special precautions in each individual case.

Both methods for the partition of the nucleic acids have been adapted to the analysis of very small amounts of tissues (milligrams of wet tissue). Steele, Sfortunato & Ottolenghi (117) and Dackermann & Patterson (118) described an ultramicromethod based on Schneider's procedure, whereas Davidson, Leslie & Waymouth (119) applied the principle of the Schmidt-Thannhauser procedure to the determination of DNA and RNA in tissue cultures. A new nephelometric procedure for the determination of P (0.1–7 μg.) as strychnin-phosphomolybdate which has been described by Bergold & Pister (120) might be of value for the nucleic acid determinations in very small amounts of tissue.

The method of Schmidt & Thannhauser has also been used to study the incorporation of radioactive phosphorus into the nucleic acid fractions. All investigators who used the method for this purpose [Villee *et al.* (121), Davidson *et al.* (122), Jeener (123), Friedkin & Lehninger (124)] discovered that the highest radioactivity is found in the very small fraction of the inorganic phosphate formed after incubation with sodium hydroxide ("phosphoprotein" phosphate fraction). This fraction has been found in isolated nuclei of rabbit liver and bird erythrocytes by Davidson & McIndoe (125). The complete separation of this fraction from the ribonucleic acid phosphate is difficult; its presence does not appreciably affect the figures of ribonucleic acid phosphate, but it interferes with determinations of the P^{32} content of the ribonucleic acid. Jeener (123) recommends, therefore, the isolation of the ribonucleotides according to Hammarsten (126) as the lanthanum salts. The alkali labile protein phosphate fraction has been designated as the phosphoprotein phosphate fraction by Schmidt & Thannhauser. It should be pointed out, however, that the only basis for this term is the fact that no other protein bound phosphate is so far known to be transformed into inorganic phosphate by alkali treatment. Whereas the interpretation of the phosphate figures obtained in both nucleic acid fractions is supported by other independent methods (determinations of the carbohydrate and of the purine groups), no possibility for such controls exists at present for the phosphorus figures of the "phosphoprotein" fraction.

Quantitative histochemical methods for the determination of nucleic acids in single cells and nuclei.—Ever since Caspersson (127) initiated the application of histochemical spectrophotometry for the determination of nucleic acids

in single cells and intracellular structures, attempts have been made to eliminate optical (128, 131) as well as chemical (132) sources of errors[4] by the use of experimental standardization methods [Pollister & Ris (128)]. A thorough investigation on histochemical standardization procedures has recently been carried out by Ris & Mirsky (134). Although the specific object of this work was the histochemical determination of DNA by means of the Feulgen reaction, the calibration methods of Ris & Mirsky (see following paragraph) will be applicable in principle to other histospectrophotometric procedures.

Differential histochemical determinations of desoxyribonucleic acids and ribonucleic acids: (*a*) *DNA.*—The applicability of Feulgen's nucleal stain to quantitative histochemical determinations of DNA has been investigated by Stowell (135), by Di Stefano (136) and by Ris & Mirsky (134). The latter measured the light absorption of nucleal stained nuclei of cells which could be obtained in the form of uniform suspensions (nucleated red blood cells), and which therefore permitted the comparison and the calibration of the histochemical absorption data with the values obtained by quantitative chemical analysis of the cell suspension. Cells of known DNA content were then mounted on a slide together with the cells or sections whose DNA contents were to be determined. Thus, the "standard" cells and the unknown cells were exposed under the same conditions to all manipulations necessary for the spectrophotometric determinations. The DNA content of the unknown cells was calculated from the proportions of the extinction coefficients and from the known DNA content of the "standard" nuclei.

(*b*) *RNA.*—The histochemistry of RNA offers greater difficulties for quantitative interpretations than that of DNA, since RNA is usually detected by the decrease of ultraviolet absorption or of the basophily observed after treatment of the section with ribonuclease. It has been emphasized that the ribonuclease preparations must be free of proteolytic activity (137, 138, 139). A convenient nephelometric method for the determination of proteolytic activity in enzyme preparations has been described by Chow & Peticolas (140). Erickson, Sax & Ogur (141) reported that the treatment of the sections with ribonuclease can be successfully replaced by the use of perchloric acid solutions at room temperature. Under these conditions, ribonucleic acid is completely removed from the sections, whereas the desoxyribonucleic acid-containing structures remain stainable. This is explained by the well known fact that mild acid hydrolysis of DNA leads to the formation of acid insoluble hydrolysis products of the type of thymic acid. Perchloric acid has no appreciable absorption in the ultraviolet range. The author of this review feels that the replacement of ribonuclease by specific chemical reagents would be very desirable in the histochemistry of nucleic acids.

[4] Observations of Bradfield & Errera (133) suggest caution in the interpretation of quantitative data concerning the ultraviolet absorption by structures of living cells. They found that the intensities of the ultraviolet absorption in living paramecia and red blood cells change considerably under the influence of the irradiation used during the spectrophotometric measurements.

Vendrely-Randavel (142) and Pouyet (143) used 1 *N* hydrochloric acid at 60° for 10 min. for the same purpose.

Staining with methyl green, which was introduced as a specific chromatin staining dye by Unna (144) and which, according to Brachet (145) permits—in combination with pyronin—the histological differentiation of DNA and RNA, has likewise been used for the histochemical differentiation of DNA and RNA. According to Pollister & Leuchtenberger (146), the affinity of DNA for methyl green is decreased after treatment of sections with reagents which lead to a depolymerization of DNA (hot water) even though no appreciable amounts of DNA are extracted. Kurnick (147) attributes the differential staining of DNA and RNA with methyl green-pyronin to the higher degree of polymerization of DNA as compared to that of RNA.

Descriptions of optical equipment for such determinations can be found in papers by Thorell (129), Pollister & Moses (148) and Mark & Ris (149). Pollister, as well as Mirsky, has pointed out that the application of histochemical spectrophotometric data to the quantitative determination of nucleic acids is at present limited to objects of spherical shape (spherical nuclei, nucleoli). Commoner (130, 131) pointed out that the computation of such data on the basis of Beer's law is only possible if the absorbing particles have random orientation. This prerequisite is not always met in cell preparations used for spectrophotometric analysis; in particular, denaturing influences seem to favor orientation of nucleoprotein molecules (130).

Properties of Nucleic Acids

Composition.—The composition of nucleic acids has been one of the main topics during the past two years. Some examples of the results obtained by the partition of nucleic acid hydrolysates with the help of recent analytical procedures are given in Table I. The figures of the table demonstrate the general validity of the results obtained with chromatographic procedures, since the values for the purines for which reliable older methods are available agree with the results obtained by earlier investigators. For example, the difference between the guanine to adenine ratio in pancreas and yeast ribonucleic acid confirms the results obtained by Levene & Jorpes many years ago. Some values reported are of a preliminary nature insofar as they are assumed to reflect the composition of the nucleic acid samples analyzed, since the problem of the quantitative hydrolysis of the nucleic acids and of the prevention of partial destruction of the bases has not yet been entirely solved. This applies particularly to the pyrimidine components, but Chargaff *et al.* (150) observed that even the purine groups of some desoxyribonucleic acids of bacterial origin are comparatively resistant under conditions which readily permit their quantitative liberation from animal desoxyribonucleic acids.

Apart from these obvious reservations, the importance of the recent analytical methods can hardly be overestimated. They permit the investigation of certain nucleic acids which can be prepared in minute amounts

only, and which for this reason could not have been analyzed with older procedures.

Radiation effects.—Stern (152) found that solutions of desoxyribonucleinates are highly sensitive to high intensity electron irradiation. Considerable decreases of viscosity (depolymerization) were observed whereas the light

TABLE I

Nucleic acid	Molar proportion of bases*					Nucleic acid P accounted for: per cent	Authors
	Adenine	Guanine	Cytosine	Uracil	Thymine		
RNA Yeast	1	1	0.63	0.7		95	Chargaff *et al.* (93)
RNA Yeast	1	0.95					Hotchkiss (76)
RNA Pig's pancreas	1	2.7	0.95	0.45		86	Chargaff *et al.* (93)
RNA Pig's liver	1	1.7	1.4	0.7		89	Chargaff *et al.* (93)
DNA Thymus (calf)	1	0.8	0.6		0.95	90	Chargaff *et al.* (150)
DNA Spleen	1	0.8	0.67		0.93	88	Chargaff *et al.* (150)
DNA av. tubercle bacillus	1	2.4	2.2		0.9	76	Vischer *et al.* (151)
DNA Yeast	1	0.55	0.55		1.05	75	Vischer *et al.* (151)

* The proportion of purine and pyrimidine bases are given as ratios with adenine taken as one; in one preparation of yeast RNA, Loring *et al.* (98) find a cytidine: uridine ratio of one.

absorption remained unaltered. According to Sinsheimer & Hastings (153), uracil and uridine are particularly sensitive toward radiations in certain ranges of the ultraviolet. It was found that both lose a considerable part of their characteristic ultraviolet absorption after irradiation. Under certain conditions (subsequent irradiation with visible light) these alterations were reversible.

Molecular weight.—Jungner *et al.* (154, 155) measured the dielectric constants of very dilute nucleate solutions in electrical fields of varying frequencies for the approximate computation of the molecular weights. The values obtained in the presence of small concentrations (0.002 *M*) of sodium chloride indicate a much smaller average particle size than those found in the absence of sodium chloride. This effect of sodium chloride was

completely reversible. The salt effect just described is paralleled by that on other physicochemical properties of DNA, such as the viscosity which is strongly decreased in dilute salt solutions [Jordan (156)]. Jungner *et al.* (154) assume that only the particles with the smallest molecular weight are real polymers in which the nucleotide groups are interlinked by covalent bonds, whereas in larger particles existing in salt free solutions the polar groups of different particles are loosely associated with each other.

Jungner *et al.* (154) used the dielectric data not only for the determination of the relative particle sizes of the DNA molecules in solutions with different salt concentrations, but also for the computation of the molecular weights of DNA. They arrived at much lower values for the average molecular weight (135,000) of highly polymerized DNA preparations than those calculated from sedimentation and diffusion data (more than 500,000). It is obvious, however, that the absolute interpretation of dielectric data for highly asymmetric molecules involves some arbitrary assumptions and must be considered as preliminary as long as comparative data on polymers of similar chemical properties and of known average particle size are not available.

An important general contribution concerning the separation and physicochemical characterization of polydisperse solutions of high polymers has been made by Claesson (157). He succeeded in fractionating such solutions by chromatography. The analysis of the fractions was used for the calculation of the weight distribution function of such solutions. The application of Claesson's technique might be helpful in the study of polydisperse polynucleotide solutions.

Titration.—The titration of nucleic acids has been considered as the principal tool for the approximate estimation of the amount of phosphoric monoester groups (secondary phosphoryl groups) in the nucleic acid molecule. All recent titration curves reveal a sharp difference between yeast RNA and highly polymerized DNA. The alkali consumption between pH 5 and pH 8 (range of secondary phosphoryl groups) by yeast ribonucleic acid is equivalent to approximately 25 to 30 per cent, in DNA only to approximately 5 per cent of the total amount of phosphorus groups. Recent observations of Schmidt, Zöllner, Hecht & Thannhauser (158) suggest that the high alkali consumption of RNA between pH 5 and pH 8 is largely caused by buffering effects which are not related to the phosphoric acid groups. These observations are in essential agreement with the results of methylation experiments reported by Barker (159) and by Anderson, Barker & Farrar (160), who found that methylation of yeast nucleic acid results in the uptake of eight methyl groups for four mononucleotide groups. Four of these methyl groups are alkali labile ester groups. This suggests that the amounts of secondary phosphoryl groups are much lower than those calculated from the alkali uptake in the range from pH 5 to pH 8.

Cosgrove & Jordan (161) titrated highly polymerized DNA from lamb thymus and from herring sperm. A short exposure to pH values of 2.5 and of 12 caused the appearance of new groups titrable between 2.0 and 6.0 and between 9.0 and 12.0. These groups are interpreted as amino and as imino-

carbonyl groups present in hydrogen bonded form in the genuine nucleic acid and liberated under the pH conditions mentioned above. A similar behavior was earlier described for DNA from calf thymus by Gulland, Jordan & Taylor (162). The results of the British authors have recently been confirmed by Signer & Schwander (54).

The electrophoretic mobility of highly polymerized DNA from calf thymus was studied by Creeth, Jordan & Gulland (163). The substance migrated in a monodisperse manner; the mobility (1.4 μ per sec. per volt per cm.; ionic strength 0.2) increased only very slightly with increasing concentration (despite a 10-fold increase of the viscosity) but decreased greatly with increasing ionic strength. The curve of the mobilities, as plotted against the pH, closely resembled the titration curve. After exposure in solutions of pH values beyond pH 5 and pH 10 (see above), no appreciable changes in the mobility were observed. The charges of DNA in solutions of various ionic strengths have been determined by Creeth & Jordan (164) by measurements of membrane potentials. Data concerning the flow birefringency and the viscosity of desoxyribonucleate solutions have been reported by Schwander *et al.* (165, 166, 167).

Metachromasy.—Nucleic acids differ from other basophilic cell constituents (mucopolysaccharides, metaphosphate) inasmuch as they do not show metachromasy unless the staining is very strong (high ratio dye:nucleic acid). This phenomenon has been analyzed spectrophotometrically by the late Michaelis (168) and by Lison (169). Since metachromasy is most likely caused by the association of dye molecules to polymers, the interpretation of the particular behavior of nucleic acids is correlated with the question of why staining of nucleic acids prevents association of the adsorbed dye molecules.

An extensive study on the protective effect of nucleates and mononucleotide ions on inorganic colloids has been carried out by Neuberg & Roberts (170).

Ohlmeyer (171) found that nucleic acids form complexes with acid prostate phosphatase at pH 3.7. The enzymatic activity is strongly but reversibly inhibited in these complexes; it is fully restored by increasing the pH. No inactivation takes place in the presence of sufficient amounts of other proteins.

Nature of the Linkage Between Nucleic Acids and Proteins

The dissociation of typical natural nucleoproteins such as thymonucleohistone in concentrated salt into protein and nucleate solutions renders it unlikely that both components are linked by covalent bonds. On the other hand, nucleoproteins differ from diffusible salts because both components remain associated in solutions of low ionic strength. A similar problem is encountered in the association of protein molecules to micelles. On the basis of measurements of volume changes during ionization or during proteolysis of proteins, Jacobsen & Linderstrøm-Lang (172) came to the conclusion that there is no evidence which would justify the assumption of hydrogen bonds

as structural forces in protein micels. This work does not refer to nucleoproteins, but it might have an important bearing on future work concerning the linkages between nucleic acids and proteins in their natural complexes.

Distribution of Nucleic Acids in Cells

Amount of DNA per cell.—Remarkable regularities concerning the amounts of DNA per cell have been observed by Boivin,[6] Vendrely & Vendrely (173), Vendrely & Vendrely (174, 175, 176), and by Mirsky & Ris (177), who determined the amounts of DNA per nucleus in various tissues of several species (beef, chicken, mouse, rat, some marine animals). It was found that the amounts of DNA per cell were constant in various types of somatic cells of a given species, but that these values were different in different species. The mature egg and sperm cells contained smaller amounts of DNA than the somatic cells; in many species the amount of DNA in these cells was half of that present in the somatic cells. The constancy of the amount of DNA per cell is in striking parallelism to that of the number of chromosomes per cell of a given species and strongly suggests an essential relation of DNA to the genes, in contrast to the views of Stedman & Stedman (178). The amounts of DNA found by Vendrely & Vendrely and by Mirsky & Ris in the nucleus are in agreement with the figures obtained by Davidson & McIndoe (125) in nuclei of fowl erythrocytes and rabbit liver.

It should be emphasized that in their preliminary communication, Mirsky & Ris (177) stressed the fact that they found in some cases considerable deviations from the simple quantitative relations just described. It appears, therefore, as advisable to use caution in the interpretation of the important observations just discussed until more extensive data are available, particularly in view of the fundamental nature of the biological problems involved.

Nucleus.—Ris & Mirsky (179) demonstrated that even in the resting nucleus the structures of the chromosomes are present and that desoxyribonucleic acid is not dispersed throughout the whole nucleus but is present exclusively in the chromosomal structures. The localization of DNA in the chromatin network of the resting nucleus has been studied by Calvet, Siegel & Stern (180) with the help of the electron microscope.

Leuchtenberger (181) observed that pycnotic nuclei of degenerating tumor cells stain weaker with methyl green than do the spherical nuclei of viable cells, despite similar DNA contents. She concluded that, in the pycnotic nuclei, the DNA was less highly polymerized than in spherical nuclei.

Nucleolus.—Pollister & Leuchtenberger (137) reported new evidence that the nucleic acid present in the nucleoli of meiotic nuclei of sporocytes of *Zea* maize is exclusively ribonucleic acid. They found that after digestion

[6] During the past year, nucleic acid chemistry lost an outstanding investigator in the person of André Boivin who died on July 10, 1949, in Strasbourg. A review of his life work has been given by M. Javillier in an address to the Académie des Sciences in Paris (1).

with protein-free ribonuclease (139), the decrease of the absorption in the ultraviolet range was similar to that observed after extraction with hot trichloroacetic acid, which dissolves both types of nucleic acid.

Boivin *et al.* (143, 182) found that nucleoli of cells of higher animals lost their affinity for basic dyes (such as toluidine blue) after treatment with ribonuclease or hydrochloric acid, but could still be stained with iron and hematoxyline. Thus, the staining with iron hematoxyline indicates the presence of structural elements other than nucleic acids in the nucleoli.

Cytoplasmic structures.—The staining properties of mitochondria are affected by ribonuclease or hydrochloric acid in a manner similar to that just reported for the nucleoli (143, 144, 182), whereas the microsomes lose their affinities to both toluidine blue and iron hematoxyline under these conditions. According to Hogeboom (183) 27 per cent of the RNA of rat liver homogenates is present in the mitochondria and 57 per cent in submicroscopic particles. Schneider (184) and LePage & Schneider (185) found likewise that the submicroscopic particles of liver homogenates of rats and rabbits contained a much higher percentage of the total RNA of the homogenates than the mitochondria.

The presence of RNA in certain basophilic cytoplasmic granules which appear under pathological conditions, particularly during acute infections, has been demonstrated by Rich & Berthrong (186).

Determinations of nucleic acid in tissues.—According to Mandel & Métais, (187) the total nucleic acid phosphorus content of human plasma is normally between 4.4 and 6.5 mg. phosphorus per l. The nucleic acid of plasma is predominantly RNA. No significant changes have so far been found in diseases. (Values for patients with tumors have not been reported.) Human red blood cells contain exclusively ribonucleic acid (56 mg. ribonucleic acid phosphorus per l. of erythrocytes). Fowl erythrocytes contain 500 mg. ribonucleic acid phosphorus and 1.4 gm. desoxyribonucleic acid phosphorus per l. of erythrocytes [Mandel, Métais & Bieth (188)].

Clavert *et al.* (189) found that striated rat muscle contains 22 mg. ribonucleic acid phosphorus per 100 gm. fresh tissue. They demonstrated that the RNA is localized in the anisotropic segments of the muscle fibers and is responsible for the basophily of these segments. The degeneration of the muscle after section of the nerve is accompanied by a strong decrease of its RNA content.

Bieth, Mandel & Stoll (190) found that during the development of the brain of the chick the amounts of RNA increase faster than those of DNA.

Leblond *et al.* (284) used a histological autograph method in order to study the localization of newly formed DNA in tissues after the injection of P^{32}-phosphate into the rat.

The Nucleic Acid Content of Tissues under Different Functional Conditions

Starvation.—Despite the constant DNA content per cell, the amount of DNA per weight unit of chromosomes varies very considerably in different

somatic cells of the same species and in different conditions of a given tissue. This is not caused by the variation of DNA, but of other constituents (for example proteins, lipids) of the chromosomes. Any discussion concerning the relative amounts of nucleic acids per weight unit of nuclear material, however, must be considered as preliminary for technical reasons. Whereas the present methods of isolating nuclei permit the quantitative recovery of DNA (134), it is certain that they entail considerable and uncontrollable losses of other nuclear constituents such as proteins. Pollister & Leuchtenberger (191) have demonstrated such losses by comparing the results of cytochemical protein determinations in the nuclei of tissue sections and in isolated nuclei. Lamb (192) has criticized the identification of isolated nuclear threads with chromosomes.

Mirsky & Ris (177) found that the amounts of DNA per weight unit of liver chromosomes are higher in starving than they are in well fed animals. In the cells so far investigated the amount of DNA per gram of chromosomes is smaller in cells with abundant cytoplasm than in those with small amounts of cytoplasm. This would mean that other (probably metabolic) functions of the nucleus, besides those of the genes, are localized within the chromosomes. The observations demonstrate the close metabolic relations between nucleus and cytoplasm. Direct histological evidence of an exchange of ribonucleic acid between nucleus and cytoplasm under certain conditions has recently been reported by Altmann (193). Marshak (194) studied the degradation of P^{32}-labelled nucleoproteins during the autolysis of isolated liver nuclei. He concluded that nuclei contain a precursor of DNA and nuclear and cytoplasmatic RNA. Mandel, Mandel & Jacob (195) reported that during fasting the total amount of RNA of a liver of the animal decreases very considerably, whereas the amounts of DNA remain unchanged. Similar observations were made in muscles during the atrophy caused by the section of the sciatic nerve.

Campbell & Kosterlitz (196, 197) studied the effects of choline-deficient diets and those of protein-free diets on the amounts and the rates of turnover of liver nucleic acids. Deficiency of choline was practically without effect; in protein-free diets a loss of ribonucleic acid was observed which was accompanied by an increased rate of incorporation of inorganic phosphate into the RNA fraction.

Growth.—Davidson & Leslie (198) and Leslie (199) found that during the later stages of vigorous growth of fibroblast cultures (seven days) from chicken hearts the amounts of DNA in the cultures increase at faster rates than RNA, protein and phospholipides. In earlier experiments with larger individual cultures, in which the metabolic effects of the growth of some cells were probably in part compensated by the necrosis of others, strong increases of RNA and of the ratio RNA to DNA were observed by Davidson, Leslie & Waymouth (200). Villee *et al.* (121) demonstrated in experiments with P^{32} that the large amount of RNA is not the precursor of DNA synthesis during the development of the fertilized Arbacia egg. According to Fraenkel-Conrat & Li (201), the cessation of growth of hypophysectomized

rats is paralleled by a decrease of the turnover rates of thymus and liver nucleic acids. Injection of estradiol into pigeons was followed by significant increases of the amounts of DNA per liver and by strong increases of the amounts of RNA and of phosphoprotein per liver. [Mandel & Mandel (202)]. Similar observations on the nucleic acid fractions of the liver of pregnant rats have been reported by Campbell & Kosterlitz (203). Peterman *et al.* (204) observed a very considerable increase of the ribonucleic acid content of leukemic (AKm strain) spleen of mice as compared to that of normal animals. The increase involved mainly the submicroscopic particles of the cytoplasm and the nuclei, whereas the larger granules and the mitochondria remained unaffected. No increase of the DNA content was observed. Characteristic cytological differences between the RNA-containing structures of normal glandular epithelium of the uterus and between those of cancers of the endometrium were observed by Atkinson *et al.* (205).

Hochberg & Hydén (206) studied the histochemical changes which accompanied the gradual degeneration of the motor nerve cells in the anterior horn of the spinal medulla. They reported correlations between the amounts of cytoplasmic and nucleolar nucleoproteins and between the paralytic symptoms occurring at various intervals after the experimental occlusion of the abdominal aorta of rabbits.

Nucleic Acids During the Growth of Bacteria and of Viruses

Di Carlo, Schultz & Fisher (207) found that the RNA content of various yeast strains and its behavior under varied conditions of growth is characteristic for each strain; in general, increased aeration was accompanied by a decrease of the RNA contents of the yeast. No general correlation with the nitrogen content of the medium and the RNA content of various yeast strains could be established.

The formation of large amounts of RNA during the lag phase has been demonstrated by Morse & Carter (208) for *Bacterium coli* and by Leonardi (209) for other microorganisms.

Hedén (210) studied the yields of T_2 bacteriophage in different stages of growth of the host. He found that maximal yields per bacterium were not obtained during the logarithmic phase of growth but during the lag phase, which is characterized by a peak in the amount of ultraviolet absorbing substances (chiefly ribonucleic acid). It appears possible that the nucleic acid content of the host is related to yield of the phage. Price (211) obtained increased yields of *Staphylococcus muscae* phage by adding a ribonucleoprotein-containing fraction of yeast to the medium. The fraction had no influence on the growth of the host.

Enzymes

Uricase.—Altman, Smull & Guzman-Barron (212) reported a new procedure for the partial purification of uricase from autolysates of beef kidney. The enzyme is adsorbed on a zinc hydroxide gel from the dialyzed autolysate

and eluted with 0.1 *M* phosphate at pH 7.5. The eluate is purified by a second adsorption with an aluminum hydroxide suspension which leaves the uricase in the supernatant.

According to Pretorius (213), British Anti-Lewisite (BAL) has no inactivating effect on uricase. The zinc content of uricase preparations can be greatly diminished by BAL without appreciable decrease of their enzymatic activities.

Nucleoside-phosphorylases (*nucleosidases*).—Friedkin, Kalckar & Hoff-Jørgensen (214) demonstrated that the cleavage and synthesis of purine desoxyribonucleosides is a reversible phosphorolysis comparable to that of the purine ribosides. [Kalckar (215)]. Desoxyribose-1-phosphate has been isolated in the form of its crystallized cyclohexylamine salt and is even less acid-stable than ribose-1-phosphate. [Kalckar (216)]. Determinations of inorganic phosphate in the presence of this ester can only be carried out with magnesia mixtures. The enzyme is present in acetone-dried rabbit liver.

Manson & Lampen (217) found that the enzymatic cleavage of thymidine in bone marrow (first observed in Thannhauser's laboratory by Deutsch & Laser (218) and by Klein (219)) is likewise a reversible phosphorolysis and that purine and pyrimidine nucleoside phosphorylases are different enzymes

Wajzer & Baron (220) reported observations suggesting the enzymatic transformation of ribose-1-phosphate to ribose-5-phosphate as well as the enzymatic condensation of ribose-3-phosphoric acid with certain specific purines to form the corresponding nucleoside-3-phosphates.

Nucleotidases.—The mononucleotidases (phosphatases) are reviewed in detail in the chapter on nonproteolytic enzymes.

Ross & Ely (221) found with histochemical methods that highly polymerized nucleic acid is hydrolyzed by alkaline intestinal phosphatase only at very high substrate concentrations; in corroboration of earlier observations by other authors, it was found that the rates of enzymatic dephosphorylation increased parallel with the degree of depolymerization of the substrate. Ek, v. Euler & Hahn (222) found that intestinal phosphatase, which had been inactivated by dialysis, is partly reactivated by the addition of a boiled extract of intestinal mucosa.

Bamann *et al.* (223) made the interesting observation that in lanthanum salts of glycerophosphate, the ester linkage is so unstable that spontaneous formation of inorganic phosphate occurs during the standing of solutions of lanthanum glycerophosphate at room temperature.

There is accumulating evidence that alkaline phosphatase is a constituent of all investigated specimens of cell nuclei. Richter & Hullin (224) found alkaline phosphatase in nuclei isolated from cerebral cortex, a tissue which contains practically very little alkaline phosphatase in its non-nuclear components. The recent observations of Meyerhof & Green concerning the transphosphorylating properties of alkaline phosphatase (mononucleotidase) (225) suggest new possibilities for the biological significance of phosphatases in cells and tissues.

Polynucleotidases: (*a*) *Ribonucleases.*—An unexpected specificity has been

found for crystalline ribonuclease by Kerr, Seraidarian & Wargon, (226) and by Schmidt, *et al.* (227). This enzyme is practically inactive towards pancreas nucleic acid under optimal conditions for the hydrolysis of liver ribonucleic acid (227) and yeast ribonucleic acid. Since pancreas nucleic acid disappears rapidly during the incubation of minced pancreas, Schmidt *et al.* conclude that this organ contains a second ribonuclease which hydrolyses pancreas ribonucleic acid.

Cerlotti (228) found that streptomycin has no inhibitory effect on the action of ribonuclease. According to Jones, Stacey & Webb (229), the first stage of autolysis of *Streptococcus citreus* (B_9) is caused by a bacterial ribonucleinase.

(*b*) *Desoxyribonucleases.*—According to Zamenhof & Chargaff (230) prolonged digestion of DNA with crystallized pancreas desoxyribonuclease splits the largest part of the nucleic acids into dialyzable fragments; however, an undialyzable fraction remains which differs by its higher purine to pyrimidine ratio from the original desoxyribonucleic acid.

Weissman & Fisher (231) correlate the activating effect of magnesium ions on desoxyribonuclease with their depressing effect on the viscosity of desoxyribonucleate solutions. They conclude on the basis of kinetic data that magnesium ions alter the substrate so that the enzyme may function. A specific inhibitor of pancreas desoxyribonuclease was found by Dabrowska, Cooper & Laskowski (232) in the extracts of rapidly growing crop glands of pigeons during the later part of the brooding period. The inhibitor is a protein and occurs only in small amounts in resting glands.

LePage (233) reported that the concentration of nucleic acids in homogenates of Flexner-Jobling rat carcinoma decreases during incubation due to the action of enzymes, but that the initial concentrations are maintained in the presence of ATP.

According to Mazia (234) the desoxyribonuclease content of the Arbacia embryo does not increase during the first 40 hr. of development. The enzyme obtained from unfertilized eggs cannot be sedimented in a centrifugal field at 20,000 *g* within 15 min., but the largest part of the enzyme becomes sedimentable after 40 hr. development.

Friedenwald & Cromwell (235) found with histoenzymatic methods that in rat kidney the enzyme system which dephosphorylated DNA is mainly located in the nucleus whereas RNA is hydrolysed in the cytoplasm as well as in the nucleus.

Desoxyribonuclease of bakers' yeast has been studied by Zamenhof & Chargaff (238). It hydrolyses animal and yeast DNA at similar rates. It is specifically inhibited by a protein fraction of the yeast which does not affect pancreas desoxyribonuclease (210).

(*c*) *Depolymerases of metaphosphates.*—Ingelman & Malmgren (236) observed with viscosimetric methods that extracts of molds and yeasts are capable of depolymerizing high molecular polymetaphosphates. The pH optimum for the depolymerase activity of *Saccharomyces cer.* is 7.2, for that of various species of penicillium between 4.5 and 4.8. Hultin (237) has

deduced a formula for the calculation of the enzymatic activities of depolymerases from viscosimetric data.

Enzymes of ribose metabolism.—Dische (239) found that the incubation of nucleosides with hemolyzed red blood cells results in the disappearance of the pentose reaction and in the formation of triose phosphates and hexosephosphates. Since the hemolysates were deprived of ATP, it is concluded that the reaction products originated from the enzymatic transformation of the ribose group of the added nucleosides, probably from its enzymatic cleavage by an aldolase. The same enzyme system is probably responsible for the metabolic transformations of nucleosides observed by Waldvogel & Schlenk (240). They described the isolation of hexose-6-phosphate (equilibrium ester) from digests of D-ribose-5-phosphate or ribonucleosides with cell free liver preparation. Since the origin of the equilibrium ester of hexoses from glycogen could be excluded, it was evidently formed from the action of enzymes on ribose-5-phosphate. According to Rapport, Canzanelli & Guild (241) the enzymatic phosphorylation of adenosine in kidney homogenates does not lead to the formation of adenylic or inosinic acids. Probably, this phosphorylation proceeds according to a mechanism similar to those just described.

Cohn & McNair-Scott (242) reported evidence for the reverse sequence of enzyme reactions, that is for the enzymatic formation of ribose-5-phosphate from hexose phosphates. They obtained from yeast and from *E. coli* cell-free enzyme solutions which formed ribose-5-phosphate from 6-phosphogluconic acid in the presence of triphosphopyridine nucleotide (TPN) and of phenazine. The individual phosphoric acid esters, as well as the dephosphorylated sugars, were separated and identified by means of chromatographic methods.

Deaminases.—The presence of powerful deaminases of adenine, guanine, and cytosine in cell free extracts of yeast and of *E. coli* has been reported by Chargaff & Kream (243). Hermann & Josepovits (244) found that the largest part of the 5-adenylic acid deaminase in muscle is bound to myosin and can be demonstrated in this protein fraction even after repeated recrystallization.

Metabolism of Nucleic Acids

The following references are intended as supplements to the organized presentation of the recent work on nucleic acid metabolism given in last year's volume by Davidson (245).

Biological synthesis of purines.—Greenberg (246) has studied the utilization of C^{14}-labelled formic acid for the synthesis of hypoxanthine in pigeon liver homogenates. Besides hypoxanthine, another radioactive reaction product could be detected by paper chromatography, whereas the formic acid had almost completely disappeared.

Excretion of purines.—Fisher, Algeri & Walker (247) determined spectrophotometrically the urinary excretion of caffeine after oral administration. The excretion starts promptly, but only 5 per cent of the ingested drug appeared in the urine within the first 24 hr. The presence of ribonucleotides

and of easily (7 min.) hydrolysable phosphate esters in the urines of patients with progressive muscle dystrophy was demonstrated by Minot, Frank & Dziewiatkowski (248).

Uric acid metabolism.—Karlsson & Barker (249) reinvestigated the distribution of labelled atoms in the uric acid obtained from the excreta of pigeons after ingestion of various labelled precursors. Their results agreed essentially with those of Buchanan, Sonne & Delluva (250), with the exception that the methylene group of glycine appeared not only as a precursor of carbon in position 5, but to appreciable extents in positions 2, 4, and 8. Bendich, Geren, Bodansky & Brown (251) studied the fate of 1,3-N^{15}-labelled uric acid in man. During four days, following oral ingestion of 150 mg. uric acid, 80 per cent of the radioactivity was recovered from the urine, one third of this amount in the form of uric acid and one half in the form of urea. In earlier similar experiments with rats only negligible amounts of N^{15} were found in urinary urea after ingestion of N^{15} uric acid (252).

Benedict, Forsham & Stetten (253) calculated from the urinary excretion of injected 1,3-N^{15}-labelled uric acid that the metabolic pool of uric acid in normals is approximately 1.2 gm., but that it is much higher in patients with gout. Urinary ammonia and urea contain some N^{15} after injection of 1,3-N^{15}-uric acid. The relative excess of the rate of uric acid formation over that of its excretion was computed to be approximately 20 per cent.

Incorporation of purines and pyrimidines into nucleic acids.—The observations of Brown *et al.* (254) that ingested adenine but not guanine can be utilized as precursor for both purines in tissue nucleic acids have stimulated much further work on the turnover of tissue nucleic acids. Bendich & Brown (255) found that, besides adenine, synthetic 2, 6-diaminopurine can be utilized as precursor of the guanine group of nucleic acids. It is very interesting that, according to Burchenal *et al.* (256), 2, 6-diaminopurine (see page 151) considerably increases the survival time of mice with acute lymphatic leukemia strain AK_4. Reichard (257) has investigated the question as to whether or not adenine can be generally considered as the precursor of nucleic acid guanine. He studied the effect of injected N^{15}-glycine on the N^{15} distribution in RNA and DNA in tissues. After the chromatographic separation of adenine and guanine, the total amount of N^{15} in the bases was partitioned into that of the amino groups and that of the purine rings by deamination of the purines. Reichard concludes from his results that adenine might be the precursor of guanine in the RNA fraction of some tissues, but that in other cases the formation of the guanine group of nucleic acids, particularly that of DNA is independent of adenine.

Further evidence in favor of the formation of the guanine groups of nucleic acids from precursors other than adenine was reported by Bergstrand *et al.* (258). These investigators injected N^{15}-glycine into rats after partial liver hepatectomy and determined the amounts of N^{15} incorporated in the individual bases of the nucleic acid fractions of the nuclei and the cytoplasm of the regenerating livers. Since the atom per cent excess N^{15} was higher in guanine than in adenine and higher in uridine than in the purines, it can be

concluded that under these conditions adenine was not the precursor of the newly formed guanine groups of the nucleic acid fractions. Similarly, uridine could not have originated from the purines.

Labelled cytosine (16), hypoxanthine and xanthine (259) were found to be ineffective as precursors of the purine bases of tissue nucleic acids.

Roll, Brown, Di Carlo & Schultz (260) studied the incorporation of purine and pyrimidine groups into the tissue nucleic acids of rats after ingestion of N^{15}-labelled yeast ribonucleic acid or injection of a nucleotide mixture obtained from it by alkaline hydrolysis. The bound purines were incorporated to a somewhat lesser extent than that observed with free adenine. Contrary to the behavior of the free pyrimidines, incorporation of the bound pyrimidines was observed. In good agreement with these results are observations of Hammarsten, Reichard & Saluste (261) who found that after injection of N^{15}-labelled pyrimidine nucleosides (obtained from N^{15}-RNA) considerable amounts of N^{15} appear in the pyrimidine groups of the ribonucleic acids of rats. The linkage with ribose, however, is not a prerequisite for the incorporation of injected pyrimidines into the nucleic acids of the rat. Bergström *et al.* (262) found that after injection of N^{15}-orotic acid (uracil-4-carboxylic acid) (6.1 atom per cent excess N^{15}), the cytosine and uracil groups of the ribonucleic acid contain N^{15} in amounts of 0.87 and 1.1 atom per cent excess respectively, whereas the purines contained only negligible amounts of N^{15}. According to Reichard (263), orotic acid can act as precursor not only for RNA pyrimidines, but also for DNA pyrimidines.

Kidder *et al.* (264, 265) carried out metabolic studies on the animal microorganism *Tetrahymena geleii*, which requires an exogenous source of purines for growth. This requirement can be met by guanine compounds, but not by adenine compounds, although they have a sparing effect. Thus, the purine metabolism of this organism differs from that of mammals. Addition of 5-amino-7-hydroxy-1H-v-triazolo[d]pyrimidine caused a powerful inhibition of growth which could be overcome by the addition of guanine compounds to the medium. The inhibitory action of this compound is therefore a competitive antimetabolic effect. The corresponding triazolo pyrimidine in which the 5-position is unsubstituted was without effect.

Abrams, Hammarsten, Reichard & Sperber (266) studied the metabolism of RNA in "nitrogen starved" yeast (*Torulopsis utilis*) after placing it in a medium which contained N^{15}-ammonium ions. The rapid assimilation of nitrogen was accompanied by a rapid turnover of the purines of the nucleic acid fraction, whereas the amount of RNA did not increase.

Nucleic acid derivatives as growth factors for microorganisms.—The important role of nucleic acid components as growth factors for microorganisms can only be briefly mentioned in this review. The requirements of purines and pyrimidines by Neurospora mutants have been further studied by Fairley & Loring (267). Pearson (268) isolated a mutant strain of *Photobacterium fischeri* which requires for growth one of the purines, xanthine, hypoxanthine, guanine or adenine, or guanine or guanylic acid. Uric acid,

caffeine, or the nucleic acid pyrimidines cannot replace the growth promoting purines. A mutant strain of *E. coli* with similar requirements for purines has been described by Guthrie (269). Falco, Hitchings, Russel & Van der Werff found that 2,4-diaminopyrimidines are antagonists of purines and of pteroylglutamic acid in the metabolism of *L. casei* (270). Several lactic acid bacteria require desoxyribonucleosides (271) for growth on a defined basic medium; others require thymine desoxyribonucleoside (271) specifically. In some lactic acid bacteria, desoxyribonucleosides (272), especially thymidine (in relatively large doses) and vitamin B_{12} (273, 274) can replace one another; in others, vitamin B_{12} cannot replace the desoxyribosides (275, 276). It appears that the action of vitamin B_{12} is connected with the enzymatic synthesis of desoxyribonucleic acid, possibly of desoxyribose. The fact that desoxyribose itself has so far been found to be without effect (271) does not exclude this possibility since its 1-phosphoric acid ester rather than the free sugar is in all probability the intermediary of the biological degradation of the nucleosides.

Mutagenic desoxyribonucleic acid fractions: (Transforming factors).—Hotchkiss (277) has reinvestigated the possible presence of proteins in the mutagenic DNA fraction isolated from pneumococci. He demonstrated that the small amounts of amino acids appearing in acid hydrolysates of this fraction cannot be attributed to the presence of proteins in the mutagenic DNA fraction because they consisted practically exclusively of glycine and originated from the decomposition of adenine during the acid hydrolysis.

Taylor (278) demonstrated the presence of a second transforming principle in the desoxyribonucleate fraction of Type III, smooth pneumococci. One of the factors (present in "rough" and "smooth" strains) is capable of transforming the "extremely rough" variant into the "rough" variant, the other (present only in "smooth" strains) is capable of changing the rough form into the smooth form. The effects of each factor are inhibited by desoxyribonuclease if the enzyme is added to the transforming cultures during the first 5 hr. of incubation.

Pharmacological effects of nucleotides and nucleosides.—The pharmacological effects of injections of pentose nucleotides on normal and tumor bearing animals have been studied by Barakan, Barker, Gulland & Parsons (279). [Concerning the pharmacological effects of some synthetic nucleosides see (30)]

Effects of irradiation.—Hévesy (280) demonstrated that the incorporation of C^{14}-labelled acetate into the purines of the total nucleic acids of various tissues is markedly reduced after irradiation with x-rays. Similar effects of x-ray irradiation were observed by Holmes (281), who studied the incorporation of P^{32} into the nucleic acid fraction of rat tumors. Marshak (282) reported similar effects of x-ray irradiation on rat tissues, but found an increased uptake of P^{32} into the nucleic acids of the nuclei of mouse lymphoma after irradiation with small doses of x-rays.

A new technique for the study of irradiation effects is based on the use

of radioactive isotopes. In particular, the incorporation of P^{32} into the desoxyribonucleic acid fraction offers interesting mutagenic possibilities due to the localization of DNA in the chromosomes. Arnason (283) studied the histological alterations of the chromosomes of seedlings grown in solutions containing nonlethal concentrations of P^{32}.

Kelner (285) made the interesting observation that several microorganisms (*Streptomyces griseus, E. coli, Penicillium notatum, Saccharomyces cerivisiae*) recover from otherwise lethal ultraviolet irradiation after subsequent short irradiation with visible light below wave lengths of 5,100 Å. Dulbecco (286) reported reactivation of ultraviolet inactivated *E. coli* bacteriophage by visible light.

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CARBOHYDRATE METABOLISM[1]

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A number of stimulating and valuable papers have appeared in the field of carbohydrate metabolism. Contributions on the intermolecular transfer of phosphate catalyzed by "phosphomutases" and phosphatases in the absence of the adenylic system, mark a notable advance. The importance of determining the pace maker in complex chain reactions has gained additional emphasis from an analysis of poorly glycolyzing tissue preparations. The wide gap between the behavior of isolated enzymes and complex cell particles is being slowly bridged by a growing appreciation of the unique properties of double-headed enzymes and other multi-enzyme systems. Phosphorylation coupled to hydrogen transport from pyridine nucleotide to oxygen, and citrate synthesis catalyzed by a soluble enzyme system are among the achievements of the year. The use of pure hormones can perhaps be expected to eliminate some of the difficulties of interpreting the rôle of the endocrines in the regulation of carbohydrate metabolism.

The usual abbreviations have been used: ATP and ADP for adenosinetri- and disphosphate, TPN and DPN for tri- and diphosphopyridine nucleotide, TCA for tricarboxylic acid, and OSA and OAA for oxalosuccinic and oxaloacetic acid, respectively.

Isolated Enzyme Systems

Enzymes of glycolysis.—The substrate specificity of hexokinase in mammalian tissues has been a frequent subject of speculation. Fructose phosphorylation has been observed by Vestling *et al.* (1) in homogenates obtained from rat liver which were unable to phosphorylate glucose. By fractional separation Cori & Slein (2) have shown that a glucokinase and a fructokinase are present in rabbit muscle and liver. Direct evidence of this type is still lacking for brain. The problem has been studied by Meyerhof & Wilson (3) and Wiebelhaus & Lardy (4). Both laboratories found that at high ATP concentrations the affinity of the brain enzyme for glucose was very much higher than for fructose. At high substrate concentrations, when glucose and fructose were phosphorylated at approximately equal rates, various inhibitors were found to inhibit each of these to a different degree. While in itself this cannot be construed as evidence in favor of two enzymes, other data showing variations in the relative activity of glucose to fructose phosphorylation in different preparations, lend support to the possibllity. On the other hand summation of activity in the presence of more than one substrate has not been observed (5, 6). In an interesting study employing

[1] This review covers the period approximately from December, 1948 to January, 1950.

extracts of acetone dried beef brain, Harpur & Quastel (6) have shown that the phosphorylation of D-glucosamine is apparently catalyzed by brain hexokinase, the affinity of the amine for the enzyme being about the same as that of fructose, though the rate of phosphorylation is much lower. That hexokinase is involved is suggested by the observations that glucosamine inhibits competitively the phosphorylation of glucose and fructose and that N-acetylglucosamine, which is not acted upon itself, inhibits the phosphorylation of all three sugars to varying degrees. The same authors have shown (7) that the inhibition of choline acetylation by glucose and fructose in brain extracts is due to competition for ATP by the two ATP requiring systems, hexokinase and choline acetylase. If a high level of ATP is maintained, acetylation then proceeds in the presence of the sugars.

Previous investigations on the nature of the inhibition of glycolysis by L-glyceraldehyde, have pointed to hexokinase as the site of inhibition (8, 9). Rudney (10) found that a higher concentration of glyceraldehyde was required to inhibit glycolysis in extracts than in slices of the corresponding tissue and that an even higher concentration was required to inhibit the hexokinase activity of a partially purified yeast preparation. The more recent work of Lardy (11) has shown that L-sorbose-1-phosphate is probably the actual inhibitor. This compound exerts a 50 per cent inhibition on hexokinase at $7 \times 10^{-6} M$ and has no effect on phosphohexokinase or phosphohexose isomerase. Glycolyzing tissues are capable of converting L-glyceraldehyde to L-sorbose-1-phosphate by condensation with dihydroxyacetone phosphate, a reaction which is catalyzed by aldolase, as shown by Meyerhof *et al.* (12). A comparison by Lardy of crude extracts of yeast, tumor, kidney, and liver, subjected to various treatments, reveals that preparations which are not inhibited by glyceraldehyde fail to bring about the condensation. Since, according to Lardy's mechanism of inhibition, glyceraldehyde is converted to sorbose-1-phosphate, the susceptibility to glyceraldehyde should be related to the aldolase content of the preparations. The observations quoted above appear to support this view and it is probable that the lack of agreement which characterizes the earlier literature on this subject can be reconciled in terms of the rate of sorbose-1-phosphate formation in the various preparations investigated.

Barker & Lipmann (13) found that glucose, arabinose, glycerol, and erythritol were rapidly phosphorylated anaerobically by ATP in propionic acid bacteria. Sorbitol and mannitol were handled somewhat differently, in that oxidation to a sugar probably precedes phosphorylation.

An investigation of galactokinase prepared from galactose-adapted yeast has been conducted by Wilkinson (14). It was found that galactose-1-phosphate is the product of phosphorylation, that magnesium ion is required in addition to ATP and substrate, that galactose-6-phosphate can be excluded as an intermediate, and that the enzyme is distinct from hexokinase. These results, obtained independently, are in entire agreement with the earlier report of Trucco *et al.* (15). The latter group have made an intensive study of the point at which galactose metabolism joins the main stream of

glycolytic reactions. With partially purified galactokinase, prepared from the lactose fermenting yeast *Saccharomyces fragilis*, the formation and accumulation of galactose-1-phosphate results from galactose. However, by employing a crude extract supplemented with glucose-1,6-diphosphate, there occurred a further transformation to glucose-6-phosphate. With a modified enzyme preparation Caputto, Leloir *et al.* (16) then succeeded in separating the following steps: galactose-1-phosphate $\overset{(a)}{\rightleftarrows}$ glucose-1-phosphate $\overset{(b)}{\rightleftarrows}$ glucose-6-phosphate. Step (b) is phosphoglucomutase-dependent, while step (a) requires, in addition to an enzyme, a thermostable factor found in commercial yeast and mammalian liver. Identification of the cofactor may elucidate the mechanism of the inversion of carbon 4 brought about in step (a).

Another pathway of galactose utilization has been considered by Totton & Lardy (17) in an investigation of tagatose-6-phosphate metabolism. Since tagatose bears the same relation to galactose as fructose does to glucose, the theoretical possibility exists that were D-tagatose-1,6-diphosphate capable of being split by aldolase, the same two trioses would be formed as normally arise from fructose diphosphate. To this end the metabolism of synthetic D-tagatose-6-phosphate (the preparation of which is described) was investigated in phosphohexokinase containing extracts of brain and tumor. While appreciable rates of phosphorylation and triose phosphate formation were observed, comparable to that obtained with fructose-6- or glucose-6-phosphate, the authors realize that the significance of these findings depends upon eventual proof of the biological conversion of galactose to tagatose phosphate. The enzymatic transformations of glucose-6-phosphate and mannose-6-phosphate to fructose-6-phosphate are each catalyzed by a separate enzyme in rabbit muscle (18) and in yeast (19).

In connection with studies of the rôle of glucose-1,6-diphosphate in the phosphoglucomutase reaction discussed below, a new enzyme has been isolated from yeast and rabbit muscle by Leloir and his collaborators (20). The enzyme, glucose-1-phosphate kinase, catalyzes the reaction: glucose-1-phosphate + ATP → glucose-1,6-diphosphate + ADP. Although, like hexokinase, it catalyzes a phosphorylation in the 6 position, it is not identical either with hexokinase or phosphohexokinase but displays remarkable substrate specificity in that all the naturally occurring phosphorylated hexoses except glucose-1-phosphate are inert. Purified enzyme, free of phosphoglucomutase and hexokinase, has been obtained from rabbit muscle. With this preparation glucose diphosphate is formed equal to one-half of the acid labile phosphate of ATP in accordance with the above reaction. While this mechanism appears to represent the pathway of glucose-1,6-diphosphate synthesis in animals and yeast, the di-ester can be formed from glucose-1-phosphate in partially purified extracts of *E. coli* without the addition of ATP, apparently by transphosphorylation between two molecules of glucose-1-phosphate (21). This interesting reaction represents a type of transphosphorylation reaction predicted by Lipmann several years ago (22).

Outstanding contributions to the action of "phosphomutases" have been made this year by several laboratories. Since the original proposal of Leloir *et al.* (23) that glucose diphosphate is the coenzyme in the phosphoglucomutase catalyzed conversion of glucose-1-phosphate to glucose-6-phosphate, a considerable body of supporting evidence has been presented by the same group of investigators. Employing an enzymatic assay procedure based on activation by the coenzyme, they obtained the compound 70 per cent pure by a difficult isolation from crude hexose diphosphate and have very ingeniously shown the structure to be that of glucose-1,6-diphosphate. Taking advantage of the extreme acid lability of the 1-phosphate group, they isolated a monophosphate ester after acid hydrolysis which was identified enzymatically and chemically as glucose-6-phosphate (24). According to their hypothesis, the apparent intramolecular phosphate shift is accomplished by an interesting and novel mechanism of coenzyme participation: glucose-1,6-diphosphate + glucose-1-phosphate ⇄ glucose-6-phosphate + glucose-1,6-diphosphate. The 1-phosphate of the coenzyme is transferred to the 6 position of glucose-1-phosphate in the forward reaction while the 6-phosphate is transferred to the 1 position of glucose-6-phosphate in the back reaction. By this means the coenzyme is continuously being converted to substrate and being regenerated by substrate (24, 25).

Posternak (26) has succeeded in obtaining pure synthetic α and β-glucose-1,6-diphosphates and found interesting differences in the acid lability of the 1-phosphate group in these compounds and in α-glucose-1-phosphate. Sutherland, Cohn, Posternak & Cori (25) found that the β form is completely inactive as coenzyme for phosphoglucomutase, while the α form exhibits a very high enzyme affinity with a K_m of $5 \times 10^{-7} M$. Examination of many natural and synthetic preparations of glucose-1-phosphate revealed a wide variation in coenzyme content ranging from zero to 0.1 per cent. The latter concentration would provide an excess several times over the optimal requirement, an observation which explains why this factor has, until recently, remained undiscovered. Sutherland *et al.* (25) have thoroughly investigated the mode of participation of glucose diphosphate in the phosphoglucomutase reaction by means of doubly labeled glucose-1-phosphate containing P_{32} and C_{14}. On incubating this compound with crystalline enzyme and non-isotopic glucose-1,6-diphosphate, equilibration of the labeled phosphate and labeled glucose between glucose-1-phosphate, glucose-6-phosphate, and glucose diphosphate was attained. The exchange of both glucose and phosphate can most readily be explained by the mechanism indicated above. Jagannathan & Luck (27, 28), however, on the basis of an observed exchange between phosphate labeled glucose-1-phosphate and the nondialysable phosphate of a highly purified enzyme, suggest a mechanism involving a transfer of phosphate between substrate and enzyme. While the crystalline preparation employed by Sutherland *et al.* (25) was phosphate free, there is evidence in the report of Cardini *et al.* (24) that some preparations contain bound coenzyme. Two other activating factors of phospho-

glucomutase, magnesium ion and cysteine, have also been extensively investigated. The enzyme is quite sensitive to certain heavy metals (27, 29, 30). Sutherland (30) has related the activation by cysteine and by a number of other substances including proteins, to their metal binding properties, an explanation which can perhaps also be applied to the sulfite activation observed by Jagannathan & Luck (27).

Investigation of the nature of the phosphate shift in phosphoglyceric acids by Sutherland, Posternak & Cori (31) has shown that the conversion of 3-phosphoglyceric acid to 2-phosphoglyceric acid by phosphoglyceric mutase is activated by 2,3-diphosphoglyceric acid in catalytic amounts. This compound was isolated in 1925 by Greenwald (32) from red blood cells. In an experiment with labeled D-3-phosphoglyceric acid containing P^{32} and an excess of nonlabeled, 2,3-diphosphoglyceric acid, exchange of the phosphate group between the mono and di-ester was observed (31). These authors formulate a mechanism of phosphate transfer quite analogous to that for phosphoglucomutase.

Baranowski, who originally described myogen A (33), has reexamined the question of its identity with aldolase (34). The following facts, instructive to enzymologists and enzyme purists, emerge from his investigations and from the history of the dispute surrounding this problem. Engelhardt (35) was the first to report that myogen A preparations had a high aldolase activity. Subsequently, Meyerhof & Beck (36) compared the aldolase activity of myogen A with the highly purified rat muscle aldolase of Warburg & Christian (37). Since the myogen A preparation had only one-sixth of the rat aldolase activity, Meyerhof & Beck concluded that myogen A was not identical with aldolase. Then Taylor *et al.* (38), by a very simple fractionation method, prepared crystalline aldolase from rabbit muscle which was readily converted into typical myogen A crystals. They concluded that aldolase is identical with myogen A although their preparations were only half as active as rat aldolase prepared by Warburg & Christian. Now, Baranowski & Niederland (34), working in Cori's laboratory, have succeeded in explaining how everyone concerned in this dispute has been right. By using the preparative method of Taylor *et al.* (38), they obtained an aldolase from rabbit muscle equal in activity to Warburg's rat aldolase. This preparation, when recrystallized at pH 5.6, gave typical myogen A crystals. Following the original method (33) for myogen A, they obtained aldolase of 15 per cent activity, confirming the findings of Meyerhof & Beck (36). This crystalline myogen A preparation also contained α-glycerophosphate dehydrogenase. Despite the above evidence of heterogeneity, the crystalline material was found to be homogeneous in the ultracentrifuge and at several pH values electrophoretically. The presence of contaminating enzymes with a high turnover number such as lactic or α-glycerophosphate dehydrogenase, appears to be a sensitive criterion of protein heterogeneity.

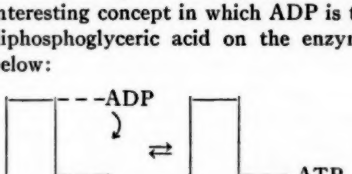
Baranowski (39) has prepared crystalline α-glycerophosphate dehydrogenase from rabbit muscle. The equilibrium constant for the DPN catalyzed

reaction: dihydroxyacetone phosphate$\rightleftarrows\alpha$-glycerophosphate was found to be 1.4×10^4 at 22°C. and pH 7.0. The turnover number of the α-glycerophosphate dehydrogenase is reported to be 26,500 at 20°C. This figure probably represents a minimal value, since the measurements were carried out in the absence of protecting proteins. Addition of serum albumin or gelatine protects α-glycerophosphate dehydrogenase and other enzymes which undergo rapid surface denaturation (40).

Important contributions in the field of transphosphorylations have been made by Bücher (41) and by Meyerhof & Oesper (42). The two reactions studied:

(*a*) 1,3-diphosphoglyceric acid + ADP $\rightleftarrows$ 3-phosphoglyceric acid + ATP
(*b*) phosphoenolpyruvic acid + ADP $\rightleftarrows$ pyruvic acid + ATP

represent the energy transfer reactions in glycolysis between the carbohydrate fragments and the adenylic acid system. While measuring the enzyme-substrate dissociation constants of the various components of reaction (*a*), Bücher made the observation that ATP did not compete with ADP in the forward reaction but rather with 1,3-diphosphoglyceric acid. Stimulated by these experimental observations, Bücher developed an interesting concept in which ADP is transferred to the 1-phosphate of 1,3-diphosphoglyceric acid on the enzyme surface as shown in the scheme below:



PPGA = 1,3-diphosphoglyceric acid

PGA = 3-phosphoglyceric acid

The equilibrium constant for reaction (*a*) was found to be 3.3×10^3 and will be discussed together with reaction (*b*). Meyerhof & Oesper (42) studied the equilibrium constant of reaction (*b*) and obtained a value of $K = 2 \times 10^3$ which is close to that of reaction (*a*). From these data the calculated $\Delta F°$ is $-4,700$ calories for reaction (*a*) and $-4,300$ calories for reaction (*b*). An energy content of 16,000 calories, derived from the above data for the enol bond of phosphopyruvic acid and the acyl bond of 1,3-diphosphoglyceric acid, are in excellent agreement with previously calculated values (43).

By incubating common hexoses and inorganic phosphate with preparations of intestinal phosphatase for several days, Meyerhof & Green (44) succeeded in demonstrating phosphate ester synthesis and in measuring the equilibria. The calculated $\Delta F°$ values for reactions involving phosphate ester synthesis are $-3,000$ calories for glucose-6-phosphate and galactose-6-phosphate, $-2,700$ calories for fructose-1-phosphate and mannose-6-phosphate, and $-2,200$ calories for α-glycerophosphate. Meyerhof & Oesper (42) point to difficulties in obtaining accurate values for the equilibrium

constant from rate measurements of the forward and back reaction. With the use of C^{14}-labeled pyruvate, a value for $K = k$ synthesis/k hydrolysis was obtained. This value for K, calculated from the velocity constants, was found to be unreasonable, and the lack of reliability of this method was ascribed to differences in affinity of the reacting components to the enzyme.

An attempt to correlate the equilibrium constant with the maximal velocities (V_1 and V_2) of the forward and back reaction and with the Michaelis substrate affinity constants has been formulated by Horecker, Kornberg & Colowick (45). In the equations

$$A \rightleftharpoons C; \qquad\qquad A+B \rightleftharpoons C+D$$

$$K_{eq} = \frac{K_C}{K_A} \times \frac{V_{1max}}{V_{2max}}; \qquad K_{eq} = \frac{K_C}{K_A} \times \frac{K_D}{K_B} \times \frac{V_{1max}}{V_{2max}}$$

K_A and K_C represent the Michaelis constants for the substrate and product of the reaction respectively. In the case of more complex reactions involving reduction of coenzymes such as DPN or TPN, the product of the affinity constants is inserted, K_D and K_B representing the K_m for reduced and oxidized nucleotide. Data and calculations on yeast alcohol dehydrogenase by Negelein & Wulff (46) are in excellent agreement with the above equation. In the case of the more complex reaction (*a*) studied by Bücher (41), the experimental value of 3.3×10^3 is about five times higher than expected from the above formula. This is perhaps due to the effective competition of the 1,3-diphosphoglyceric acid with the two components of the back reaction, ATP and phosphoglyceric acid, as explained by Bücher's scheme.

Enzymes of polysaccharide metabolism.—The use of isotopes has proved of considerable value in studying the mechanism of the hexose unit transfer in phosphorylase systems. Cohn (47), using O^{18}-labeled inorganic phosphate has ingeniously shown with both muscle and sucrose phosphorylase that during the transfer reaction, the cleavage of glucose-1-phosphate, which may be C—O$\vdots$—P or C$\vdots$—O—P, occurs between carbon and oxygen. It is interesting that the position of cleavage is such as to retain the energy of the anhydride bond in the glucose residue. This is perhaps to be expected from a consideration of the glucose transferring reactions which these enzymes catalyze. On the other hand, employing O^{18} water, it was found that with alkaline and acid phosphatases, the cleavage occurred between the oxygen and phosphorus bond. This type of cleavage should retain the energy in the phosphate residue in the presence of a suitable acceptor and may account for the recent observations of Meyerhof & Green (44, 48) who found that alkaline phosphatase catalyzes a direct transfer of phosphate from glucose-1-phosphate to fructose or glycerol. There was no exchange with P^{32}-labeled inorganic phosphate.

It has been previously reported that crystalline phosphorylase from

rabbit muscle degrades approximately 80 per cent of glycogen (49). Hestrin (50), in confirmation of unpublished experiments by G. T. Cori, has now obtained by repeated recrystallization a purified phosphorylase, which degrades branched polysaccharides only 40 per cent. A factor responsible for the degradation of glycogen beyond the branch points was removed during recrystallization. The limit dextrin formed from glycogen after action of pure phosphorylase can be hydrolyzed 24 per cent by β-amylase. This treatment destroys its effectiveness as a primer of glycogen synthesis by muscle phosphorylase. These results suggest that the end-groups on the primer, which remain after phosphorylase action, are at least three glucose units long. New purification procedures for muscle phosphorylase (51, 52) and studies of its prosthetic group have been reported (53, 54). Changes in the relative contents of phosphorylase *a* and *b* in muscle after starvation have been studied (55).

Doudoroff *et al.* (56), in independent investigations, studied the fermentation of maltose by *E. coli* and confirmed the observations of Monod & Torriani (57). The enzyme amylomaltase, which the latter investigators described and purified, catalyzes the reversible reaction maltose$\rightleftharpoons$polysaccharide + glucose (56, 58).

An amylosucrase preparation obtained from extracts of *Neisseria perflava* by Hehre (59), catalyzes the synthesis of a branched polysaccharide from sucrose. As in the case of amylomaltase, inorganic phosphate does not participate in the reaction. Dextran formation from dextrin in *Acetobacter viscosum* was described by Hehre & Hamilton (60), and the vitamin requirements for dextran synthesis in *Leuconostoc* were studied by Whiteside-Carlson and Carlson (61, 62).

The properties of crystalline α-amylase from germinated barley were studied by Schwimmer & Balls (63). The use of starch as adsorbent in the purification procedure was further discussed in a second paper (64). The pure enzyme does not contain inositol which previously had been reported to be an active constituent of pancreatic α-amylase (65). Pancreatic amylase was investigated by Mindell *et al.* (66) with respect to extent of substrate hydrolysis and rates of glucose and maltose formation in the presence of different starches.

Enzymes of oxidative metabolism.—Another enzyme system of the TCA cycle has been obtained in soluble form by Stern & Ochoa (67, 68). They have demonstrated the formation of citrate from acetate, ATP and OAA by soluble enzymes from *E. coli* and tissue extracts. An analysis of this reaction revealed the presence of at least two enzymatic components, one required for the "activation" of acetate and one for the condensation of the 2 carbon intermediate with OAA to form citrate. As predicted by Lipmann (69) and demonstrated by Olson *et al.* (70) for homogenates of pantothenic-acid deficient animals, the partially purified system requires coenzyme A for citrate formation. The lack of aconitase activity in these preparations excludes *cis*-aconitate and *d*-isocitrate as primary condensation products. The acetate

activating enzyme was found to be most active in bacterial extracts, while the condensing enzyme was readily obtained from extracts of animal tissues, including brain.

More than 20-fold purification of aconitase has been achieved by Buchanan & Anfinsen (71), principally by the use of alcohol fractionation. The estimated purity of this preparation is 30 per cent, with isocitric dehydrogenase, fumarase, and hemoglobin as readily detectable impurities. No evidence for a separation of the two aconitases was obtained, since the ratio of activity with citrate and isocitrate as substrates remained constant throughout the purification. Changes in ratio were obtained by Racker during purification (72), particularly after ammonium sulfate fractionation. Neither set of data can for the present be taken as evidence for or against the existence of two aconitases. The purification procedure of Laki & Laki (73) for the preparation of "crystalline" fumarase has been confirmed by Scott (74) and Racker (72). However, the latter investigators found that on repeated recrystallization the specific activity of fumarase became progressively lower, while a highly active, though amorphous, fraction was obtained from the mother liquor.

A DPN linked enzyme, which catalyzes the oxidation of several aliphatic and a few aromatic aldehydes, has been purified 20-fold from acetone powder extract of beef liver (75). Together with alcohol dehydrogenase, they catalyze the dismutation of acetaldehyde to ethyl alcohol and acetic acid. This explains the mechanism of "mutase" action and casts doubt upon the existence of a single mutase enzyme in liver.

An active enzyme preparation catalyzing the oxidation of lactic to pyruvic acid, glycolic to glyoxylic, and of glyoxylic acid to formic acid and carbon dioxide was described by Clagett *et al.* (76) and Tolbert *et al.* (77). By ammonium sulfate precipitation and lyophilization, a preparation was obtained from tobacco leaves with a Q_{O_2} (N) of 50,000 for glycolic acid. Lactic and glyoxylic acids were oxidized at a rate approximately one third that of glycolic acid. Since the activity ratios for these three substrates remained constant throughout the purification procedure, the authors believe that they are dealing with a single enzyme.

Multi-Enzyme Systems

In stimulating lectures, Dixon (78) stresses the value of viewing metabolism in terms of multi-enzyme systems. A clear analysis of enzymes acting in reaction chains is presented with particular reference to glycolysis and oxidative carbohydrate metabolism. Although more difficult to analyze than isolated enzyme reactions, multi-enzyme systems are probably more representative of the metabolic pattern within the cell.

Glycolysis.—The analysis of glycolytic rates in crude tissue homogenates and extracts have increased our understanding of complex chain reactions. Coenzymes and activators must be supplemented to make up for their more rapid destruction and for dilution effects in tissue preparations. The

most frequently encountered difficulty appears to be that of maintaining an optimal ATP:ADP ratio during glycolysis. As shown by numerous studies of Meyerhof and his collaborators, fermentation may cease because of an imbalance in the rate of ATP breakdown as compared to its synthesis. If ATP dephosphorylation is too slow, as with certain types of yeast maceration juice (79) hexose diphosphate (HDP) accumulates and fermentation ceases because of lack of ADP or other phosphate acceptors. This defect can be eliminated by the addition of adenosinetriphosphatase. On the other hand, if too much is present, as shown for a number of normal and tumor tissue homogenates (80, 81, 82), glucose phosphorylation ceases after a short period. In this case, addition of adenosinetriphosphatase inhibitors, such as octyl alcohol or sodium azide, help to maintain glycolytic activity. Addition of hexokinase achieves the same result. By such procedures adenosinetriphosphatase and hexokinase are "brought into step." To obtain constant rates, the adenosinetriphosphatase should be twice as active as hexokinase since twice as much ATP is synthesized as is used during glycolysis. Meyerhof & Wilson (80) found an adenosinetriphosphatase:hexokinase ratio of 8 in tumor homogenates, and addition of suitable adenosinetriphosphatase inhibitors or hexokinase were therefore required to approach the optimal ratio of 2. The rate of ATP synthesis may become the limiting factor in the presence of ferrous sulfate as shown for brain homogenates by Racker & Krimsky (83). The apparent inhibition of glucose phosphorylation is due to an inactivation of glyceraldehyde phosphate dehydrogenase which becomes the limiting factor for ATP regeneration. Addition of pure glyceraldehyde phosphate dehydrogenase fully restores the glycolytic activity. More recently the inactivation of glyceraldehyde phosphate dehydrogenase by intracellular proteolytic enzymes was shown to be counteracted by the addition of certain amino acid esters and peptides (84). The use of these small molecules as inhibitors of proteolytic enzymes may serve as a valuable tool in maintaining enzymatic activity in homogenates as well as during the purification of enzymes.

It is apparent from the above discussion that in many tissues the initial steps of glucose phosphorylation are most likely to be the pace makers in glycolysis. By using HDP as substrate in liver and tumor homogenates (85, 86), rapid lactic acid production can be obtained. However, the initial stages of glucose phosphorylation are thus omitted from the study and require separate consideration. Addition of an excess of highly purified ATP as a source of energy-rich phosphate is often unsatisfactory, as shown in the case of arginine synthesis by Ratner & Pappas (87). Excess ATP can be successfully replaced by an enzyme system which regenerates ATP from phosphoglyceric acid. Inhibition of probably another type was observed by Le Page & Potter (88) in the presence of relatively small amounts of some commercial preparations of ATP and was eliminated by further purification.

The source as well as the method of preparation of an extract may govern its fermentation pattern. Meyerhof (89) found that, in contrast to slowly dried yeast, extracts obtained from rapidly dried yeast contain considerable

adenosinetriphosphatase activity. When inhibitors of adenosinetriphosphatase are added, a typical Harden-Young reaction with accumulation of HDP is obtained. This is another example of an interruption of fermentation by an unfavorable ATP:ADP ratio.

The occurrence of individual steps of the Meyerhof-Emden scheme of glycolysis was shown in *Trypanosoma hippicum* by Harvey (90), and in *Penicillium notatum* by Lynen & Hoffmann-Walbeck (91). Glycolysis in filaria was studied by Bueding (92). Glucose utilization in blood parasitized with *Plasmodium gallinaceum* was compared to the normal rate (93). Diisopropyl fluorophosphate was reported to inhibit glycolysis in rabbit muscle (94) and carbohydrate metabolism in brain (95, 96). The inhibitory effects of naphthoquinone and of amidines on glycolysis in muscle extracts were described by Gemmill (97, 98). The effect of inhibitors on bone marrow glycolysis was studied by Lutwak-Mann (99).

Aerobic oxidations.—Although the majority of enzymes in the tricarboxylic acid cycle have been obtained and studied in soluble form, the complete oxidation of pyruvic acid to carbon dioxide and water has to date been achieved only with preparations of tissue particles. In view of the objections raised by Wood & Lorber (100) to the designation "cyclophorase" introduced by Green and his associates for these particles, the designation multienzyme particles or simply particles will be used here.

Keilin & Hartree (101), in a restudy of succinic dehydrogenase, again stress the importance of the "colloidal structure" of the particle preparations. At low phosphate concentrations, addition of denatured proteins or certain metal salts led to a marked increase of the succinic dehydrogenase activity. Observations on the activation of oxidative systems by high salt or sucrose concentrations have previously been recorded (102, 103, 104) and may point the way to an experimental "reparticulation" of enzymes in solution. Bücher's (105) studies of the disruption and recombination of a single protein (enolase), under the influence of varying salt concentration and pH variations, may serve as an additional model for such attempts. The studies of Ochoa and his collaborators (106) on the "double headed" "malic" enzyme may further our understanding of the mode of action of enzymes on particles. While the combination of malic dehydrogenase from heart muscle and oxaloacetic decarboxylase from *Micrococcus lysodeikticus* cannot carry out the oxidative decarboxylation of malic acid, the soluble "double headed" enzyme from pigeon liver rapidly catalyzes this reaction. The explanation of this phenomenon probably lies in a more efficient utilization of the intermediate substrate by the "double headed" enzyme due to the proximity of the oxidative and decarboxylative processes. A quantitative approach to the efficiency problem of cytochrome-*c* utilization was made by Slater (107) by comparing the requirement for added cytochrome-*c* with the amount of cytochrome-*c* present in actively respiring heart particles. At least 100 times greater efficiency was found in the particles.

Optimal conditions for the preparation and metabolic activity of these particles have been studied in a number of laboratories (108 to 114). Our

knowledge of the pathway of the TCA cycle is still incomplete, particularly in respect to the primary products of pyruvate and α-ketoglutarate oxidation. O'Kane & Gunsalus (115) have reported a new factor required for pyruvate oxidation in *Streptococcus faecalis*. Similarity of this factor to the factors required for growth of *Tetrahymena geleii* (116) and to a *Lactobacillus casei* factor (117) which can substitute for acetate was pointed out by Snell (118). A DPN requirement for pyruvate oxidation was demonstrated in brain homogenate by Larner *et al.* (119). This tissue appears to be particularly suited for such studies because of the rapid inactivation of DPN and TPN upon homogenation. Coxon *et al.* (111) restudied pyruvate oxidations in multienzyme particles from brain. In the absence of fumarate, considerable amounts of acetate were formed, while in its presence citrate and ketoglutarate accumulated.

Citrate has been reconsidered as an intermediate of the TCA cycle. The reason for excluding citrate was based primarily on the fact that passage through a symmetrical molecule was in apparent contradiction to isotope distribution data (120). The correctness of this reasoning was recently questioned on theoretical grounds by Ogston (121), and experimental data in support of Ogston's theory were provided by Potter & Heidelberger (122). Ogston suggested the possibility of the asymmetric utilization of a symmetrical compound and the synthesis of optically active isomers by an "asymmetrical enzyme." Moreover, the studies of Stern & Ochoa (68), demonstrating a widely distributed enzyme which catalyzes the condensation of oxaloacetate and "active acetate", point clearly to citrate or citryl phosphate as the first condensation product.

Attempts to demonstrate the existence of a TCA cycle have been made in a great number of different cells. Incorporation of isotopes into intermediates of the cycle, the accumulation of intermediates, their utilization by the metabolizing cell as well as actual demonstration of the enzymes participating in the TCA cycle, have been used as criteria. It should be emphasized that none of these approaches alone can be taken as proof of the existence of a TCA cycle but together they represent strong evidence. That the TCA cycle operates in a variety of plant species is suggested by the accumulation of succinate in the presence of malonate (123); the restoration of respiration by citrate, *cis*-aconitate and α-ketoglutarate (124); the accumulation of citrate when excised tobacco leaves are cultured on malate, the accumulation of malate when citrate is added (125, 126); the presence of isocitric dehydrogenase, oxaloacetic carboxylase and the "malic" enzyme (127, 128). The ability of bacteria to utilize substrates of the TCA cycle (129, 130) has more significance than the lack of utilization. Permeability factors may play a rôle when citrate is not utilized by *E. coli* (129) or when dicarboxylic acids are not metabolized by *Azotobactor agilis* grown on acetate (130), since extracts of both bacteria have been shown to contain an active condensing enzyme, aconitase and isocitric dehydrogenase (131). The role of the TCA cycle for the production of oxalic acid in *Aspergillus niger* is dis-

cussed by Lynen & Lynen (132) and the formation of oxalic acid from oxaloacetic and oxalosuccinic acid is suggested as an alternative to the pathway through glycolic acid oxidation considered by Smith (133). Of particular interest is the oxidation of members of the TCA cycle in purified rickettsial preparations demonstrated by Bovarnick & Snyder (134). This represents the first convincing evidence of metabolic activity in rickettsial organisms.

Advances were made in the understanding of the rôle of fluoroacetate as a respiratory inhibitor. The finding of increased citrate production, (113, 135) speaks against an effect on the initial steps of acetate metabolism. Illuminating experiments were carried out independently by Martius (136) and Liébecq & Peters (137). These investigators showed that fluoroacetate itself is probably not the inhibitor but that it is transformed into an inhibitor in the tissues. The possibility of metabolically formed fluorocitrate or fluoroisocitrate as inhibitors of isocitric dehydrogenase was suggested by Martius. An inhibitory effect of streptomycin on the TCA cycle in *E. coli* was analyzed by Umbreit (138).

Coupled phosphorylation.—Hunter (139) and Hunter & Hixon (140) made extensive studies of coupled phosphorylations. They demonstrated that washed particles of liver or kidney catalyze the following anaerobic dismutations: (*a*) α-ketoglutaric acid + oxaloacetic acid $\rightleftharpoons$ succinic acid + malic acid + CO_2 (*b*) 2 α-ketoglutaric acid + NH_3 $\rightleftharpoons$ succinic acid + CO_2 + glutamic acid. Energy-rich phosphate was trapped as hexose phosphate by the addition of glucose and hexokinase. These findings represent an important step forward in the analysis of energy yielding reactions. However, it is evident from Hunter's well controlled studies that several factors still complicate a clear analysis of the findings. On omission of either hydrogen donor or hydrogen acceptor, considerable blanks were obtained. The washed particles, therefore, still contained unknown hydrogen donors and acceptors as well as a source of free ammonia. This, and the fact that coenzymes are present in considerable quantities in well washed tissue particles (108), make the interpretation of the mechanism of the dismutation difficult. Although, as is well known, the reaction oxaloacetate $\rightleftharpoons$ malate is DPN linked, nucleotide participation in ketoglutarate oxidation has not as yet been demonstrated. Since this work suggests that DPN participates in the dismutation, it is difficult to explain why pyruvate does not substitute for oxaloacetate. If, on addition of an excess of purified lactic dehydrogenase, pyruvate remained inactive, this would suggest participation of a coenzyme other than DPN. A reexamination of the quantitative aspects of coupled phosphorylation has been published by Hunter & Hixon (114). By lowering the incubation temperature to 15° C., the phosphorylating system was preserved and experimental phosphorus:oxygen ratios of 2.5 and higher[2] were obtained with

[2] According to a personal communication from Dr. E. F. Hunter, the P/O ratio of 3.5 reported in the original paper is somewhat high.

α-ketoglutarate. Ogston & Smithies (141) on theoretical considerations have criticized the studies of Ochoa (142) who obtained phosphorus:oxygen ratios of 3 (corrected for ATPase activity). The finding of directly determined phosphorus:oxygen ratios of 2.5 by Hunter & Hixon, who excluded by a thorough analysis several possible errors due to side reactions, obviates further discussion of the objections raised by Ogston & Smithies.

Lehninger and his collaborators (143, 144, 145) have studied coupled phosphorylation in preparations of liver mitochondria prepared according to Hogeboom *et al.* (146). In the earlier experiments, esterification was followed by the uptake of P^{32}. In later experiments, ADP or AMP was used as phosphate acceptor and esterification of inorganic phosphate was measured directly. The oxidation of β-hydroxybutyrate was chosen as the most suitable DPN linked reaction because acetoacetate is not oxidized by this type of preparation. Phosphorus:oxygen or phosphorus:acetoacetate ratios approaching 2 were obtained and since there were losses due to dephosphorylation reactions, the actual ratio may have been 3. Careful experiments using radioactive tracers were carried out to exclude the possibility of pyrophosphate formation which would greatly contribute to a high phosphorus:oxygen ratio. It was conclusively demonstrated that in the mitochondria inorganic phosphate does not undergo significant incorporation into inorganic pyrophosphate. This establishes that pyrophosphate formation, which was shown to occur in the particles of Cross *et al.* (108), does not play an essential part in coupled phosphorylation. The studies of Lehninger and his collaborators constitute the first experimental evidence of coupled phosphorylation between reduced DPN and the cytochrome system. The presence of β-hydroxybutyrate dehydrogenase in the particles is a convenient feature of the system. However, it is the least well studied of the DPN-linked enzymes since it has been difficult to obtain active preparations of this enzyme in soluble form. It would be of interest to know whether oxidative decarboxylation of β-hydroxybutyrate by a mechanism similar to the "malic" enzyme can be carried out in the mitochrondria. Even if such a reaction were to occur the important conclusions drawn in this paper would not be affected. It is of special interest to learn that Lehninger (147), using reduced DPN as the sole substrate, has now succeeded in obtaining phosphorus:oxygen ratios comparable to those with β-hydroxybutyrate. Lynen & Holzer (148) studied the uptake of inorganic phosphate coupled with oxidation of butyl alcohol in yeast cells depleted of substrate. Since butyric acid, the end product of oxidation, was not further utilized and the anaerobic dismutation of butyl aldehyde did not result in phosphate uptake, the conclusion was drawn that hydrogen transfer through the flavin and cytochrome system in yeast can be coupled with phosphorylation.

The mechanism of the coupling between oxidation and phosphorylation remains unknown. A stimulating discussion of the problem has been presented by Lehninger (144) on the basis of experimental data. Two possibilities are considered: one, that two separate enzymatic pathways exist, the

first for oxidation and the second for oxidation coupled with phosphorylation; as the second possibility, the same enzyme-coenzyme system with different reactive groups is suggested. A third possibility, the formation of small molecular intermediates which undergo cyclic reduction and oxidation coupled with phosphorylation might be added (149). Compounds with a suitable potential might act as a labile shuttling system between the main large molecular components of the hydrogen transfer system and might explain the ease of uncoupling by various compounds.

Carbon Dioxide Fixation

The distribution of carbon isotope in the glucose formed *in vivo* or *in vitro* has proved a useful tool in evaluating metabolic interconversions of 3 and 4 carbon units. Shreeve, Feil, Lorber & Wood (150), replacing the less sensitive $C^{13}O_2$ by $C^{14}O_2$, have confirmed their earlier experiments (151). The isotope was incorporated by carbon dioxide fixation and equilibration into the 3 and 4 positions of glucose corresponding to the carboxyl group of pyruvate. In addition, C^{14}, amounting to 1 or 2 per cent of that found in the center carbons, was detected in the other four positions. Two possible pathways have been considered to account for the distribution in carbons other than 3 and 4. According to the first, isotope might be introduced into the methyl group of pyruvate by passage of carboxyl-labeled pyruvate through a 3 carbon symmetrical intermediate such as dihydroxyacetone. The second possibility is the introduction of isotopic carbon dioxide into the β carbon of serine, as has been shown to occur with formate (152, 153). In either case, conversion of the methyl-labeled pyruvate to a 4 carbon dicarboxylic acid by carboxylation would result in randomizing the isotope between the methyl and carbonyl carbons by equilibration through fumarate. A trip through the TCA cycle would lead to the same distribution. Koshland & Westheimer (154) and Aronoff, Haas & Fries (155) have subjected synthetic glucose-1-C^{14} to fermentation by yeast and *L. casei* respectively. Isotope was unexpectedly detected in the carbon dioxide of yeast fermentation and in the carboxyl group of lactic acid produced by *L. casei*. These findings, as well, as the very different experiments of Shreeve *et al.* (150), suggest that at some step in glycolysis there occurs a reversible formation of an unphosphorylated symmetrical 3 carbon intermediate through which isotope in carbon 1 or carbon 3 becomes equilibrated.

A number of recent papers are concerned with glycogen formation from isotopic pyruvate. A group of investigators at Harvard (156, 157, 158) have studied in liver slices the interesting effects of sodium, potassium, and calcium ions upon total carbohydrate and glycogen formation. It can be assumed from previous work that the isotope of carbonyl-labeled pyruvate appears in glucose mainly as a result of the following conversions: (*a*) direct phosphorylation of pyruvate and reversal of glycolysis will label positions 2 and 5; (*b*) reversible carboxylation of pyruvate and equilibration of dicar-

boxylic acids through furamate will randomize the isotope between the methyl and carbonyl groups of pyruvate and result in 1,6 and 2,5 labeling; (*c*) if the carboxylation occurs with labeled carbon dioxide-(derived from complete oxidation of isotopic pyruvate) followed by equilibration through fumarate, carbon 3 and carbon 4 will become marked, and (*d*) oxidation to form carboxyl-labeled acetate will also label carbon 3 and carbon 4 by condensation and oxidation reactions of the tricarboxylic acid cycle. Topper & Hastings (159), after incubating rabbit liver slices with carbonyl labeled pyruvate, found that the isotope in the glucose of isolated glycogen was equally distrubuted among carbon 2, carbon 3, carbon 4, carbon 5, and approximately two-thirds as much was found in carbon 1 and carbon 6. The principal reactions leading to incorporation of isotope in positions 3 and 4 are conversions (*c*) and (*d*) mentioned above. By these routes an isotope concentration equal to that in positions 2 and 5 was reached, owing to the relatively high rates of (*c*) and (*d*) and to the fact that dilution of respiratory $C^{14}O_2$ is low under the experimental conditions. Another demonstration of the rapidity of carboxylation appears in the results of Gould *et al.* (160), who found that isotope was incorporated into glucose to approximately the same extent, whether unlabeled pyruvate in the presence of $C^{14}O_2$ or carbonyl-labeled pyruvate was employed. With regard to the isotope in positions 1 and 6, a similar distribution, i.e., two-thirds of the amount located in positions 2 and 5, was found by Wood (161) in the glucose from liver glycogen of rats to which α-labeled lactate had been administered. Analysis of the data, for which the original papers should be consulted (159, 161) led to the estimate that four times as many pyruvate molecules have participated in carboxylation and equilibration reactions as have entered glucose directly. From the data observed with slices (159), it could also be estimated that twice as many pyruvate molecules were oxidized to completion as were converted to glucose.

The results discussed above represent the first estimation of the relative rates of carbon dioxide fixation by intact mammalian cells. The fundamental mechanism of this important process has not been clearly understood since the two possible pathways, the "malic" enzyme and the Wood and Werkman reaction, have appeared thus far to be quite different. Highly purified "malic" enzyme from pigeon liver is TPN dependent, it catalyzes the reversible oxidative decarboxylation of malate to pyruvate and carbon dioxide, and the reaction can readily be shifted toward malic acid formation and carbon dioxide fixation by a favorably linked dismutation (162, 106). On the other hand, OAA carboxylase, presumed to catalyze the fixation of carbon dioxide in OAA, has an equilibrium far to the side of decarboxylation and would therefore not be expected to be readily reversible. Fixation, as measured by incorporation of carbon-labeled carbon dioxide in OAA, employing crude or partially purified pigeon liver extracts in the presence of an excess of OAA, appears to be dependent on ATP but not on pyridine nucleotides (163, 164). Salles *et al.* (165) have shown now that isotope fixa-

tion does not occur under conditions which are unfavorable to "malic" enzyme activity, or which exclude the presence of "malic" enzyme. In crude pigeon liver extracts, with added ATP, carbon dioxide fixation in OAA occurs at pH 7.4, the optimum for "malic" enzyme, but not at pH 4.5, the optimum for OAA carboxylase. Preparations of OAA carboxylase from *M. leisodeikticus*, which are devoid of "malic" enzyme activity, as well as purified OAA carboxylase from *Azotobacter vinelandei* (166), fail to fix carbon dioxide. They (165) have shown further that carbon dioxide fixation in OAA can be brought about by the combined action of "malic" enzyme (Reaction *a*) and malic dehydrogenase (Reaction *b*) through a TPN linked dismutation between malate and OAA to give pyruvate, carbon dioxide, and malate.

$$(a)\quad C^*OOH{-}CH_2{-}CHOH{-}COOH+TPN_{ox.} \rightleftarrows CH_3{-}CO{-}COOH+C^*O_2+TPN_{red.} \qquad \left\{\begin{matrix}\text{"malic"}\\ \text{enzyme}\end{matrix}\right\}$$

$$(b)\quad C^*OOH{-}CH_2{-}CO{-}COOH+TPN_{red.} \rightleftarrows C^*OOH{-}CH_2{-}CHOH{-}COOH+TPN_{ox.} \qquad \left\{\begin{matrix}\text{malic}\\ \text{dehydrogenase}\end{matrix}\right\}$$

$$(c)\quad {}^*\text{Malate}+{}^*\text{Oxaloacetate} \rightleftarrows \text{Pyruvate}+{}^*CO_2+{}^*\text{Malate}$$

DPN linked malic dehydrogenase has been shown to react also with TPN (106). It may be seen in the sum (Reaction *c*) that a reversible decarboxylation of OAA has been accomplished indirectly through the mediation of malic acid which primes the dismutation by serving as a hydrogen donor for OAA. Salles *et al.* (165) have demonstrated clearly that isotopic carbon dioxide is incorporated in OAA in the presence of TPN, malic acid, and highly purified preparations of "malic" enzyme and malic dehydrogenase. The presence of malate was essential for fixation, and incorporation of isotopic carbon dioxide in OAA was increased at the expense of that in malate by increasing the amount of malic dehydrogenase. Since malate can substitute for ATP in crude pigeon liver extracts, it was suggested that ATP acts by promoting glycolysis from contaminating glycogen, thus supplying a hydrogen donor which is required to produce malate from OAA. Small amounts of DPN ,TPN, and glycogen have been found in these crude preparations even after prolonged dialysis. A major pathway of carbon dioxide fixation may therefore be visualized as follows: C^*O_2 and reduced TPN form isotopic malic acid which is then oxidized to isotopic OAA. In a discussion of the energetics of these reactions, Ochoa (167) points out the theoretical advantages, with respect to reversibility and efficiency, of carbon dioxide fixation via the "malic" enzyme as compared to reversible decarboxylation of oxaloacetate.

An improved preparation of parsley root OAA carboxylase has been obtained by Vennesland *et al.* (168). The rate of decarboxylation was found to follow a first order reaction under their experimental conditions. Lynen & Scherer (169) have pointed out, in a valuable general treatment of the kinetics, that spontaneous β decarboxylation follows first order, and decar-

boxylation catalyzed by enzyme saturated with substrate follows zero order reaction rates. The enzyme is widely distributed in roots, tubers, leaves, and seeds of many plants (170). More recent studies by Conn *et al.* (171) show that OAA carboxylase from parsley root and a number of other plants is associated with a TPN dependent enzyme which catalyzes the reversible decarboxylation of malic acid, and appears to be similar to the "malic" enzyme of pigeon liver described by Ochoa *et al.* (162). The presence in plant extracts of factors which rapidly destroy TPN have previously caused considerable difficulty in demonstrating "malic" enzyme activity. DPN, ATP and adenylic acid were found to exert a protective action (171). Since the protection of one nucleotide by another may lead to an erroneous impression of coenzyme specificity, the general implications of these findings can hardly be overemphasized. The effects of manganese and magnesium ions, and a number of heavy metals on the spontaneous and enzymatic decarboxylation of oxaloacetic carboxylase from several sources have been investigated by Speck (172), by Plaut & Lardy (166), and by Nossal (173). A discussion of these papers and of the rôle of metal complex formation in β-decarboxylation appears elsewhere (167).

Hormones and Related Subjects

Hormone antagonism.—Evidence for adrenal-pituitary inhibition of hexokinase activity and the reversal of the inhibition by insulin was offered by Colowick, Cori & Slein (174) in two ways: (*a*) insulin stimulated the hexokinase activity of the diabetic rat muscle in the presence of adrenal cortex extract; (*b*) the hexokinase activity of normal muscle and brain was inhibited by pituitary extract and this inhibition was counteracted by insulin. Stadie & Haugaard (175) were unable to confirm the first observation. As pointed out by these authors, no particular attention had been paid to rat strain differences. With respect to alloxan susceptibility and diabetic severity, variations in different rat strains have been observed (176). Christensen *et al.* (5) found no difference in the hexokinase activity of erythrocytes from normal, diabetic, or hypophysectomized rats; insulin and adrenal cortex extract had no effect.

The direction of carbohydrate investigations in surviving tissue have been stimulated by recent advances in the preparation and availability of two pure hormones of the pituitary. Their *in vivo* effects will be considered first. The numerous actions of pure adrenocorticotrophic hormone (ACTH) are attributed (177) to adrenal release of corticosteroids; those connected with the regulation of carbohydrate metabolism are most effectively replaced by Compound E (11-dehydro-17-hydroxycorticosterone) and Compound F (17-hydroxycorticosterone) of Kendall. The "diabetogenic" effects of ACTH, after a single administration, are manifested by a temporary glycosuria and elevated post absorptive blood sugar, as shown by Sayers *et al.* (178). On continued administration, Conn *et al.* (179, 180) have found a loss of carbohydrate tolerance and increased resistance to insulin. In the

diabetic rat, ACTH leads to increased glycosuria (181, 182). The familiar action of certain corticosteroids in promoting glycogen deposition in the adrenalectomized rat has been found to be highest with Compound E (183, 184).

It is becoming increasingly clear that the "diabetogenic" activity shown by pure growth hormone is associated with a single protein. Cotes, Reid & Young (185) have found "diabetogenic" activity in growth hormone preparations obtained free of corticotropic factor by the methods of Li *et al.* (186), Wilhelmi and collaborators (187, 188) and by their own method and judged to be homogeneous electrophoretically and in the ultra-centrifuge. De Bodo (189) found that the insulin hypersensitivity of hypophysectomized dogs was abolished by pure growth hormone, Gaarenstroom *et al.* (190) noted an increase in the glycosuria of mildly diabetic rats, and Bennett *et al.* (181, 182) found no change in the glycosuria of diabetic rats maintained on a carbohydrate-free diet. Wilhelmi *et al.* (187) reported that their purest crystalline preparations of growth hormone exhibited the highest ability to maintain a normal level of muscle glycogen in the starved hypophysectomized rat. Millman & Russell (191) found that the same preparations produced hypoglycemia in normal rats, intensified the hyperglycemia of diabetics, and increased the resistance to insulin in both groups. They are of the opinion that growth hormone exhibits contra-insulin effects in the rat when pancreatic function is impaired, but may provoke insulin excretion in the normal animal. It appears that *in vivo* manifestations of hormonal antagonism vary greatly, both with the species and experimental conditions.

Evidence of growth hormone insulin antagonism has been shown in isolated tissues. Park & Krahl (192) found that by pretreatment with growth hormone, the glucose uptake in diaphragms from normal rats was lowered to a diabetic level and the elevated rate in hypophysectomized rats was lowered to normal levels. Adrenal function, which can be replaced by gland extract, is apparently required to mediate the action of the growth hormone. Under similar conditions, Li, Kalman & Evans (193, 194) investigated the increase in glycogenesis produced by insulin. The increment was abolished in diaphragms from normal and hypophysectomized animals by previous growth hormone injection. Attention has been drawn by Stadie *et al.* (195, 196) to the combination of insulin with intact cells as a preliminary step in insulin action. Under their experimental conditions, the uptake of insulin *in vitro* by the isolated rat diaphragm appears to be under endocrine control. Insulin uptake, measured by extra glycogen formed in a glucose medium, was found to be impaired in the diaphragms of both diabetic and normal animals which were previously injected with growth hormone, but was unaffected by adrenalectomy or hypophysectomy. Though growth hormone had no effect when added *in vitro*, inhibition was found with crude pituitary extract. The effect of insulin on the rate of glucose utilization and glycogen deposition in diaphragms of normal, diabetic, hypophysectomized, and adrenalectomized rats have been reinvestigated by Villee & Hastings

(197) with C^{14}-labeled glucose. Glucose utilization was accompanied by glycogen deposition in all but the adrenalectomized animal; insulin increased glycogen formation in all diaphragms and increased glucose oxidation in all but the diabetic.

In vitro effects on glycogenesis are shown by a number of corticosteroids and appear to be somewhat lacking in specificity. Bartlett *et al.* (198, 199), using isotopic glucose, have confirmed the observations from Verzár's laboratory (200, 201, 202) that desoxycorticosterone inhibits glycogenesis from glucose, in the isolated rat diaphragm and abolishes insulin stimulation. In rat liver slices, however, with pyruvate as substrate, Chiu & Needham (203) have found that desoxycorticosterone and compound E increase the formation of total carbohydrate and glycogen.

A possible effect of insulin on coupled phosphorylation has been suggested by recent work (204, 205). These experiments are somewhat difficult to interpret since extremely low phosphorus:oxygen ratios were obtained and the increased esterification due to insulin was very small. The bearing of these results on the well known ability of insulin to stimulate pyruvate oxidation in pigeon breast muscle remains to be seen. This problem has been investigated in mammalian muscle (206, 207, 208), employing pyruvate, labeled in the carbonyl group with C^{14}. With diaphragms from diabetic rats, a decreased pyruvate oxidativation was found which could be raised to normal levels by insulin; low pyruvate oxidation in heart slices was unaffected by insulin. A connection between impaired pyruvate oxidation and restricted availability of high energy phosphate is also suggested by the acetylation studies of Hegsted and his collaborators (209, 210). They observed that the acetylation of *p*-aminobenzoic acid, a reaction known to require high energy phosphate, was reduced in diabetic rats. Acetylation was rapidly restored to normal levels by administration of ATP, by fumaric and malic acids, more slowly by insulin, and not at all by pyruvate.

Insulin and alloxan diabetes—The presence of a hyperglycemic-glycogenolytic factor in certain commerical preparations of insulin has raised the question as to whether this factor is exclusively pancreatic in origin. Sutherland *et al.* (211) have found this factor in the gastric mucosa as well as the pancreas, and in no other tissues. They have achieved a tenfold purification starting with an amorphous insulin powder as the source material. The hyperglycemic activity of a number of commercial insulin preparations have been compared by Weisberg *et al.* (212) and the effect of route of administration investigated.

It has been known for some time that insulin is rapidly destroyed in the body. Mirsky & Broh-Kahn (213) have found that aqueous extracts of a great many rat tissues display this activity to which the name insulinase has been given. The factor is heat labile, is inhibited by sulfhydryl binding compounds and appears to have the properties of an enzyme. The activity, which is reduced by fasting, seems to be related to the nutritional state of the animal (214). Destruction of insulin by the liver has been studied in the dog by Weisberg *et al.* (215).

Space permits only brief mention of a number of interesting papers in the field of alloxan diabetes. Wrenshall *et al.* (216) have correlated the insulin content of the pancreas and the degree of glycosuria with the extent of pathological change in islet cells. Further evidence that alloxan diabetes is pancreatic in origin appears from the report of Bailey *et al.* (217), who prevented diabetes in rabbits given alloxan by temporary occlusion of the arteries of the pancreas. The much more resistant guinea pig has been compared to the rat by Charalampous & Hegsted (218) in a study of the severity of the diabetes produced by alloxan, and the calf may also be included among the resistant species (219, 220). Investigations on the elevated serum alkaline phosphatase of alloxan diabetes have been continued by Cantor *et al.* (221) and an elevation of serum lipase has been reported by Tuba & Hoare (222). The list of compounds which prevent the diabetic action of alloxan have been extended to include methylene blue by Highet & West (223), and boric acid by Rose & György (224). Studies on ketosis in alloxan diabetes have been continued by Banerjee & Bhattacharya (225).

The production of experimental diabetes by alloxan has led to the suggestion that in human diabetes an alloxan-like metabolite is formed, possibly in the course of purine metabolism. Lazarow (226), has recently reviewed the experimental data supporting this theory. The reader is also referred to this valuable article for a discussion of the interesting relationship of glutathione to clinical and alloxan diabetes. In this connection, only brief mention will therefore be made of the temporary alleviation by glutathione of ACTH-induced diabetes reported by Conn *et al.* (227); of the failure by Collins-Williams & Bailey (228) to confirm a previous report by Griffiths (229) that uric acid administration induces a transient diabetes in rabbits whose blood glutathione has been previously lowered, and of the parallelism found by Grunert & Phillips (230) between sodium deficiency and low blood glutathione on the one hand, and an increased incidence of alloxan diabetes in sodium deficient rats on the other.

Methods

A simplified preparative method for TPN has been described by Le Page & Mueller (231). Several papers on amylase determination have appeared (232, 233). Sibley & Lehninger developed a method for aldolase assay in tissues (234). Dische *et al.* have devised useful methods for the specific determination of hexoses in mixtures (235). Modifications of previous tests have been described for mannitol, sorbitol, sucrose, and sorbose by West & Rapoport (236, 237). Methods for citric acid (238, 239); for lactose (240); for inulin (241); for acetic and fluoroacetic acid (242), and micro methods for glucose and sucrose (243) were described. Paper chromatography has been used for fermentation acids (244), for sugars (245), for methyl-fructoses (246), and for keto acids (247, 248).

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FAT METABOLISM[1]

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Mitochondria and Metabolism of Fatty Acids

Refinements in the methods of isolation of individual enzymes have led to an increasing knowledge of their association with certain specific cellular components, some in the insoluble and some in the soluble fractions of the cell cytoplasm or nucleoplasm. Kennedy & Lehninger (1) have shown that the mitochondria of rat liver are able to effect the complete oxidation of fatty acids as evidenced by octanoate disappearance, oxygen uptake and acetoacetate formation. Careful preservation of normal osmotic conditions must be maintained during isolation. The essential cofactors are magnesium ions, neutral salt, adenosinetriphosphate (ATP), cytochrome-*c* and catalytic amounts of malate. Oxidation of citrate, α-ketoglutarate and pyruvate plus oxaloacetate is similar to that of the Krebs cycle and condensation of oxaloacetate and pyruvate yields citrate.

Thus it is now shown that while enzymes concerned with glycolysis of carbohydrate are present in the soluble portion of the cytoplasm, all the enzymes which catalyze the complete oxidation of fatty acids are localized within one morphological unit. Specially interesting is the fact that conversion of pyruvate into the form through which it is incorporated into the Krebs cycle is associated more closely with the morphological elements of fatty acid oxidation than with those concerned in earlier steps of carbohydrate metabolism.

In an earlier paper Friedkin & Lehninger (2), using essentially the same system as reported above, showed that oxidation of L-malate by way of the Krebs tricarboxylic acid cycle, brings about incorporation of P^{32} from inorganic phosphate into phospholipid, pentose nucleic acid, and an unidentified acid-insoluble "phosphoprotein" residue, thus adding these protoplasmic constituents to the list of biological compounds which may be phosphorylated directly as the result of coupled oxidations. The authors point out that these findings increase the probability that nucleoprotein, as suggested by Spiegelman & Kamen (3), functions as transmitter of energy between catabolic reactions and protein synthesis.

Cross, Taggert, Corvo & Green (4) continued studies with their "cyclophorase system", which, as indicated by its general reactions and by the cofactors necessary for complete functioning, consists essentially of isolated mitochondria from rabbit kidney or liver. During oxidation of succinate these authors found that orthophosphate disappears to yield a phosphate ester. They employed glucose as the phosphate acceptor. Hexokinase had to

[1] This review covers the period approximately from December, 1948 to December, 1949.

be added to their more highly purified preparations. Magnesium ions, adenylic acid, hexose and substrate (in this case, succinate) were essential. Fructose and mannose could be substituted for glucose; galactose and arabinose were inactive. Fructose-6-phosphate could not serve as acceptor, indicating that phosphohexokinase was not present. The authors determined the P:O ratios during the oxidation of α-ketoglutarate, succinate, malate, oxaloacetate, pyruvate, citrate, proline and glutamate and found that from two to three atoms of phosphorus are taken up for each atom of oxygen absorbed. Gramicidin and some of its derivatives, tested in the cyclophorase system, inhibited phosphorylation without depressing the oxidation of α-ketoglutarate, as was previously reported by Hotchkiss (5) in cell-free kidney extracts.

Green (6) presented evidence that radioactive inorganic phosphorus becomes incorporated in pyridine nucleotide (DPN+TPN) and adenine polyphosphate during oxidation of all members of the citric acid cycle and of β-hydroxybutryate and glutamate by his washed kidney or liver cyclophorase system.

Kalnitsky (7), almost simultaneously, isolated the mitochondria from kidney cortex, and demonstrated that they contain the enzymes necessary for citrate formation from oxaloacetate. The yield was increased by addition of pyruvate, acetoacetate and acetate. Oxaloacetate, acting as oxidant in the medium, permitted the reaction to take place anaerobically, in confirmation of the earlier findings of Hunter & Leloir (8). Inorganic phosphate and magnesium ions were essential.

Kalnitsky (9) described a preparation of homogenized and washed kidney cortex, which, when suspended in NaCl-KCl solution in an atmosphere of oxygen, completely oxidizes acetic acid. Magnesium ions and inorganic phosphate increase the rate. This homogenate apparently contains all the essential constituents of the Krebs cycle, as lactate, caproate and succinate may also serve as substrates. Pyruvate is not oxidized (coenzyme A is probably lacking). Discrepancies between the cofactors required by this homogenate and that of the workers previously mentioned may be due to differences in the amount of washing or in the dilution of the homogenate.

Units of Fatty Acid Metabolism

Pyruvic acid-fatty acid relationship.—Although the evidence seems conclusive that acetic acid is not the active two-carbon unit of fatty acid metabolism, its metabolic behavior most nearly approximates that of the unknown intermediate, and hence it continues to be employed widely in experiments *in vivo* and *in vitro* with tissue slices and homogenates as the initial substrate in experiments in the metabolism of fatty acids. The question as to whether acetate operates through only one active form or whether more than one is possible, i.e., whether pyruvic acid is converted to the same or to a different active unit, is a problem on which some evidence has been brought out this year without, however, providing an answer as yet.

Using isotopic pyruvic acid Anker (10) obtained evidence on some of

these unsolved questions of pyruvic-acetic acid metabolism. By employing carbonyl- and carboxyl-labeled samples of pyruvic acid, he demonstrated that only carbon atoms 2 and 3 of pyruvic acid are utilized for acetylation. The same two atoms are used for fatty acid synthesis, the carbonyl carbon-atom 2, being incorporated in a random manner into the odd numbered positions of the fatty acid chain.

Anker employed two strains of rats. In one strain, the Sprague-Dawley, pyruvic acid acetylated both *p*-aminobenzoic and γ-phenyl-α-aminobutyric acids, while in his laboratory strain, only the aliphatic amine was acetylated. Both types of amines were acetylated by acetic acid. Hence, he concluded that in his laboratory strain, pyruvic acid is not converted to acetic; but since the laboratory strain of rats can utilize pyruvic acid for fatty acid synthesis, in these rats some mechanism other than conversion of pyruvate to acetate must be involved in the synthesis of fatty acid from pyruvic acid.

On the basis of Anker's computation of the isotope concentration of the metabolic pools of pyruvate and acetate under the conditions of these experiments, it was estimated that about one-sixth of the carbon atoms of the saturated fatty acids were derived from pyruvic acid, while about one-third originated from acetic acid. On the other hand, the isotope concentration of the liver glycogen was about one-thirtieth of the isotope concentration of the pyruvic acid pool, while only slight incorporation of acetic acid into liver glycogen occurred. Thus both pyruvic acid and acetic were more readily available for fatty acid synthesis than for glycogen formation. Hence dietary carbohydrate, as pointed out by Anker in confirmation of earlier observations, was more easily synthesized to glycogen than was pyruvic acid. In explanation, the large amount of carbohydrate in the diet, 73 per cent, plus that derived from 16 per cent casein, must be taken into consideration. Anker computed from the relative concentrations of the two acetylated amines in the laboratory strain of rat, that the metabolic pool of pyruvic acid averaged 6 to 7 m*M* per 100 gm. rat per day, while the acetic acid pool was calculated to be 3.5 times as great in both strains. This small size of the pyruvic acid metabolic pool brings out the fact that only a small fraction of the dietary carbohydrate can pass through pyruvic acid in its oxidation to carbon dioxide or its synthesis to fatty acids. Anker points out that the mechanism of the major part of carbohydrate metabolism thus remains unexplained. In this connection it would have been of interest to have known the amount of isotopic carbon in the respiratory carbon dioxide. Since the ratio pyruvate:acetate incorporated into saturated fatty acids differed in both strains of rats from that utilized for unsaturated, Anker concluded that these two types of fatty acids must be synthesized by different mechanisms.

Kaplan & Lipmann (11) have isolated and partially purified an acetyl phosphate, formed in the presence of extract of dried *E. coli* and high concentrations of acetate. It differs from synthetic acetyl phosphate but apparently goes over to it on short exposure to pH 1.5. This product acts as an acetyl donor in the synthesis of pyruvate in dialyzed suspensions of dried *E. coli* in the presence of excess formate. It also acts [Kaplan & Soodak (12)]

in the acetylation of sulfanilamide both in extract of *E. coli* and in pigeon liver extract and since it has already been shown [Soodak & Lipmann (13)] to be concerned in the acetylation of acetate (acetoacetate formation), at least these three processes appear to occur through the same acetyl derivative.

Stern & Ochoa (14) agree that since oxaloacetate depresses both the synthesis of acetoacetate and the acetylation of sulfanilamide, the same acetyl derivative must be involved in these reactions.

Strecker & Wood (15) in equilibria studies between carbonyl-labeled pyruvate and acetyl phosphate plus formate, concluded that the biologically produced acetyl phosphate behaves essentially like the synthetic.

Olson, Hirsch, Richards & Stare (16) have determined rates of citric acid-formation from pyruvate in the presence of fumarate and coenzyme A in the heart muscle of normal and pantothenic acid-deficient ducklings. They found a greatly depressed citrate in the deficient ducklings and interpreted this as evidence that the function of coenzyme A is involved in the initial condensation of the tricarboxylic acid cycle, with possibly an additional action upon the transformation of pyruvate to active acetate.

Relation to citric acid.—Potter & Heidelberger (17) tested the newly expressed concept of Ogston (18) that an enzyme might distinguish between the chemically identical groups of a symmetrical compound and therefore citric acid may not necessarily give rise to α-ketoglutaric acid with an equal distribution of isotope in the two primary carboxyl groups. The authors prepared citric acid from oxaloacetic and pyruvic acids by rat-liver homogenate in an atmosphere containing C^{14} carbon dioxide. The citric acid which accumulated was isolated, purified and enzymatically converted to α-ketoglutaric acid by a preparation of rat-liver homogenate. The α-ketoglutaric acid was found to be labeled solely in the carboxyl next to the carbonyl. All isolations were carred out with extraordinary precautions for attaining maximum purity. These important findings will necessitate the reexamination of other reactions in which similar deductions have been made as to the metabolic behavior of chemically symmetrical intermediates.

Stern & Ochoa (14, 19) again opened up the question as to the relation of citric acid to the condensation product of oxaloacetic acid and activated acetate. They employed an oxidizing system consisting of ammonium sulfate fractions of extract of acetone-dried pigeon liver and adenosinetriphosphate, coenzyme A and magnesium ions. The aconitase content was so low that equilibrium between the three tricarboxylic acids was not reached. The authors identified citric acid, formed possibly through citryl phosphate, as the addition product. They suggested as a possible alternative to Ogston's hypothesis, that citrate and the other tricarboxylic acids of the cycle occur in the phosphorylated forms and hence are not symmetrical.

Synthesis of Fatty Acids

Ponticorvo, Rittenberg & Bloch (20) computed that when they administered deuterioacetate at the rate of 1 m*M* per 100 gm. of weight per day, 20

per cent of the carbon atoms of the fatty acids were derived from acetate. This was figured on the basis of their previous findings that dietary acetate is diluted by acetate formed in the intermediary metabolism by a factor of 20, and that the turnover time for the total fatty acids is about nine days. Since ultimately two-carbon compounds contribute all the carbon atoms of fatty acids, these results must mean that a different two-carbon unit, supplied from the diet and from other metabolic sources, was not in equilibrium with acetate during the 158 days of the experiment; or else that longer chains than acetate, for instance, activated pyruvate, may be added in the building of fatty acid molecules and subsequently degraded to a two-carbon residue.

The mammary gland of the ruminant (goats and cows) according to Folley & French (21), in contrast to that of nonruminants (rat, mouse, guinea pig, rabbit) metabolizes acetate more rapidly than glucose. High R.Q. values, 1.20, were found for six goats and 1.06 and 1.15 for two cows, contrasted with 0.75 for six rats, indicating that within the gland of ruminants synthesis of longer chain fatty acids occurs from acetate. The same authors (22) found, however, that addition of glucose to the incubation medium caused a more rapid utilization of acetate by rabbit mammary gland slices together with an increase in the R.Q. from a mean value of 0.03 to 0.26. They suggest that glucose may act by providing glycerol for glyceride synthesis. Other explanations are possible, such as capacity of glucose to act as acceptor of high-energy phosphate bonds. Folley (23, 24) reviewed the biochemical aspects of mammary gland function, including a section on the origin of short chain fatty acids. He summarizes the evidence as indicating that milk fat may arise from three sources; (*a*) incorporation directly from the blood stream; (*b*) synthesis within the gland of ruminants from metabolites arising from the rumen, and (*c*) synthesis also within the gland from carbohydrate precursors.

Clostridium kluyverii [Stadtman, Stadtman & Barker (25)] synthesized caproic acid from carboxyl-labeled butyric acid and ethyl alcohol with the radioactivity only in the β-carbons, showing that butyric acid was not broken down to two-carbon fragments. Similarly, with carboxyl-labeled propionic acid and ethyl alcohol as substrates, β-labeled valeric was formed, and heptanoic, with the labeled carbons probably in the β- and δ-carbons. Lack of radioactivity in all the even-numbered acids synthesized showed that they were built up solely from the two-carbon substrate.

Masoro, Chaikoff & Dauben (26) studying the production of fatty acids from radioactive glucose in mice, showed an incorporation of 10 to 15 per cent of the glucose in the fatty acid fraction isolated from the whole animal after 24 hr., less than 2 per cent being accounted for in the liver fatty acids after the same interval. The specific activities of the fatty acids from liver, intestine, and carcass were in one instance, 112, 44 and 20 respectively. In eviscerated rats deprived of portal and hepatic blood supplies, the percentage of C^{14} recovered as fatty acids was of the same order as that of the normal rat, demonstrating that utilization of glucose for fatty acid formation is not dependent upon the presence of a functioning liver.

The recently renewed interest in atherosclerosis has led to a number of investigations of the metabolism of arterial tissue. Chernick, Srere & Chaikoff (27) incubated rat thoracic aortas with isotopic acetate and found an active synthesis of fatty acids. Under the conditions of their experiment about 5 per cent of the added C^{14} was recovered in the fatty acid fraction. In a further study, incubation was carried out in the presence of inorganic phosphate labeled with P^{32}. About 0.7 per cent of the P^{32} was incorporated in phospholipid in 2 hr., or 0.8 per cent per gram of tissue.

Stotz (28) reviewed the biological synthesis of fatty acids.

Oxidation of Fatty Acids

In normal mammalian tissue.—Grafflin & Green (29) investigated the oxidation of fatty acids up to *n*-tridecylic acid in the cyclophorase system and found all were oxidized except propionic. In attempting to identify possible intermediates, they found that the substitution of the β-hydroxy, α, β-unsaturated and β-keto acids corresponding to the fatty acids, gave rise to the same respective end-products. Large amounts of a "sparker," either heart extract, α-ketoglutaric or fumaric acid, or any other member of the tricarboxylic acid cycle were essential [see also Knox, Noyce & Auerbach (30)], while the parent fatty acids could be oxidized in the presence of much lower concentrations of the sparker. In a further attempt to identify intermediates, the authors reported a detailed study of caproic acid oxidation, and found β-ketocaproic acid arising from α, β-unsaturated or β-hydroxy acid, cleavage of β-ketocaproic to acetic and butyric acids, condensation of acetic (after activation) with oxaloacetic to form citric. The butyric became oxidized to acetoacetic which, upon activation, cleaved to two molecules of acetic acid. These also upon activation condensed with oxaloacetic. With a fatty acid substrate containing an odd number of carbon atoms, the residue was propionic acid, which was not further catabolized. These latter findings were confirmed through a more detailed study by Atchley (31) using the counter-current distribution method. He included the oxidation of isocaproic acid in his studies, and identified the residue as isobutyric acid, which in part gave rise to propionic acid.

Meister (32) found that while 2,4-hexadienoic acid was readily oxidized by Lehninger's preparation of liver homogenate, 2,4- and 3,5-diketohexanoic acids were metabolized at negligible rates. The author draws the conclusion that at least in this system polyketo acids are not intermediates in the oxidation of *n*-hexanoic to acetoacetic acid.

Connors & Stotz (33) announced the isolation from beef liver and the 100-fold purification of the enzyme which oxidizes triacetic to acetic and acetoacetic acids. Later, Witter & Stotz (34) in a study of rates of acetoacetate formation in various rat washed liver preparations plus a heat-stable factor from liver, found that β-hydroxyhexanoate and β-ketohexanoate were oxidized more slowly than hexanoate. Under the conditions of these more

highly purified preparations, triacetate, β, δ-diketohexanoate was not metabolized (35).

By standardizing techniques of preparing substrate solutions, Kennedy & Lehninger (36) obtained oxidation of fatty acids from C_6 to C_{18} in rat liver mitochondria preparations. Normal saturated fatty acids from C_{14} to C_{17} and oleic acid were completely oxidized, as indicated by their respiratory quotients, while with acids of shorter chains the tendency to form acetoacetate gradually increased. This eliminates the short chains as intermediates in the oxidation of the longer, and indicates some fundamental difference in their metabolism. If the first or final two-carbon unit from each acid chain had slightly greater potentialities for acetylation, a gradual decrease in acetoacetate formation would be expected as the chain increased in length. The more highly unsaturated fatty acids, linoleic and linolenic, were rapidly oxidized in this system, though it is not yet clear whether they were first reduced to oleic or stearic, or metabolized differently.

Zabin & Bloch (37) studied acetoacetic acid formation from isovaleric acid labeled in the methyl carbons with C^{13}, and in the carboxyl carbon with C^{14}. They found indication of a splitting of the isovaleric acid molecule into a three-carbon fragment, (either acetone or one giving rise to acetone,) and a two-carbon fragment which condensed to acetoacetate.

Geyer, Matthews & Stare (38) studied utilization by tissue slices, of fat emulsions with the fatty acids labeled in the carboxyl carbon. The authors point out that the rate of appearance of the labeled carbon in the respiratory carbon dioxide may be different from that which was incorporated farther back in the chain. The emulsified particles were prepared in a soybean stabilizer and were not over 2 μ in diameter. The authors compared the rate of utilization by different tissues of trilaurin with that of water soluble sodium octanoate and found in general the same pattern was followed, although slight variations were dependent upon relative concentrations of substrate. Utilization was most rapid during the first 2 hr. but continued for 4 or 5 hr. longer. Previous starvation of the rat for as much as 60 hr. had no consistent effect.

Since the administration of carbon dioxide and acetate are the basis of so many experiments, their rates of excretion are of especial interest. Gould *et al.* (39) found that 18, 42, and 45 min. respectively were required for the recovery of 50 per cent of the carbon dioxide from the intraperitoneally injected bicarbonate, acetate and succinate. At the end of the 4-hr. collection period, 95, 87 and 86 per cent respectively had been excreted as carbon dioxide. Analyses of the rats after the 4-hr. period showed isotopic glycogen in the livers following all three injections. Fatty acid and cholesterol contained no isotope after administration of either bicarbonate or succinate, but both were radioactive after acetate injection.

Dziewiatkowski, Venkataraman & Lewis (40) studied the metabolism in rabbits of branched-chain aliphatic acids represented by 2,4,4-trimethylpentanoic, 2-ethylbutyric, 2-ethylhexanoic, and hydroxyisobutyric acids.

From 35 to 87 per cent of the various acids were recovered unchanged or, in the case of the first three, as the glucuronates. They concluded that the portion not accounted for was metabolized, though no evidence was offered as to their pathways of oxidation.

The value of homogenized oils added to infusion media has received continued attention during the year, together with techniques of preparation and tests on their suitability for human injection. Contributions have been made by Shafiroff *et al.* (41), Stare *et al.*, (42, 43, 44), Lerner *et al.* (45), and others.

The use of radio-isotopic carbon has been introduced into the study of parenterally administered fat emulsions. Lerner, *et al.* (46) injected 1.5 ml. of an emulsion of tripalmitin, $C_{10}H_{21}C^{14}H_2(CH_2)_4COOH$ esterified with glycerol and dispersed to particles less than 2μ in diameter by means of supersonic energy, into 175 gm. rats. Analysis of carbon dioxide after various intervals revealed oxidation of 7 per cent after 2 hr., the interval of maximum hourly excretion, 5 to 7 per cent during the second 2-hr. period, and 57 per cent excreted by the close of 24 hr. Fifty per cent of the C^{14}-labeled fatty acid which was recovered had been incorporated into the phospholipid.

In various deficiencies.—Although pantothenic acid, one of the constituents of coenzyme A, is essential for the normal acetylation of *p*-aminobenzoic acid (PAB) in the rat, Riggs & Hegsted (47) showed that other factors affect the extent of this coupling reaction. They compared the influence of pantothenic acid deficiency with the results under thiamin and riboflavin deprivation in rats weighing about 100 gm. The thiamine-deficient rats show slightly less than normal acetylation at moderate doses of *p*-aminobenzoic acid (2.5 and 5 mg.), though normal values were obtained with smaller ones. Riboflavin deficiency lowered acetylation at all levels of PAB dosage, though less than did pantothenic acid deficiency. At 10 mg. intake of *p*-aminobenzoic acid, the effect was maximum and all deficiencies approached the same value. The amount of available acetate was not the limiting factor, nor was the amount of pantothenic acid in riboflavin deficiency, since addition of these supplements did not alter the rates of acetylation.

Villee, Pearson & Hastings (48) incubated isotopic acetate in the presence of diaphragm muscle of diabetic rats and found the production of carbon dioxide decreased to 35 per cent of the normal. Addition of insulin had no effect. They did not indicate whether this decrease was due to reduction of normal amounts of carbohydrate precursors in the diabetic, but they suggested that the primary interference might be with the condensation of acetate and oxalacetate. In diabetic heart muscle, utilization of acetate was unchanged from normal (49).

Weinhouse, Millington & Friedman (50) demonstrated that ketogenesis in diabetes in the rat cannot be ascribed to competitive inhibition of fatty acid oxidation by carbohydrates. They compared the rates of oxidation of isotopically labeled short chain fatty acids in liver slices from previously fasted rats with rates in livers of well-fed rats and found that oxidation of

the fatty acid tended to increase rather than to decrease in the latter. Pyruvate, added to the livers of the non-fasted rats further increased oxidation of acetate and butyrate. The authors turn to the more plausible explanation of inhibition of ketogenesis by carbohydrates in the increased precursors for the Krebs cycle with consequent diversion of fatty acids from acetoacetate formation to complete oxidation [see Breusch (51)].

Charalampous & Hegsted (52) found the decreased ability of alloxan-diabetic rats to acetylate *p*-aminobenzoic acid to be correlated with a relative deficiency of ATP. The latter could be replaced by acetyl phosphate (not acetate), diacetyl, dietary fat or injections of ATP, all of which the authors interpreted as supplying high energy bonds without intervention of insulin. Since diabetic animals fail to grow normally and since growth is a reflection of the availability of energy, it would be of interest to see if these supplements would increase growth.

In plants and lower animal forms.—Investigations of oxidations in plants and lower animals are of interest in determining whether the tricarboxylic system is universal in its distribution. It has been shown to be present, at least in some modified form, in nearly all types so far reported, but in only a few cases have the specific enzymes been identified.

In studying the mechanism of fixation of carbon dioxide in heterogeneous bacteria, Ajl & Werkman (53) have demonstrated that growth can occur in the absence of carbon dioxide, provided certain members of the Krebs cycle, preferably oxalacetate and α-ketoglutarate are present.

More concrete evidence is accumulating as to the coupling of phosphorylation and oxidation in tissues from many diverse forms. Barker & Lipmann (54) demonstrated the simultaneous occurrence of these processes in *Propionibacterium pentosaceum* in glycolysis and alcoholic fermentation, but the authors did not investigate this relationship in the Krebs cycle. They were unable to detect acetyl phosphate as an intermediate in the oxidative decarboxylation of pyruvate. Harvey (55), from a study of the phosphorylating systems of *Trypanosoma hippicum* found that glycolysis conforms to the Meyerhof-Cori-Embden scheme, but no evidence was obtainable for the operation of a tricarboxylic acid cycle. He suggested that this trypanosome must be dependent upon the fluids of the host to wash out the pyruvic acid that would otherwise accumulate. Massey & Rogers (56) investigated the oxidation of pyruvate and various members of the Krebs tricarboxylic acid cycle in the Nematode, *Ascaridia galli* from the chicken small intestine and *Nematodirus spathiger* and *N. filicollis* from the sheep small intestine. They found that oxygen uptake of brei prepared from whole parasites was increased by pyruvate, oxaloacetate, malate, α-ketoglutarate, fumarate and succinate. Citrate had little effect. Malonate inhibited the oxidation slightly, but the inhibition, in contrast to that of pigeon breast muscle, was increased in the presence of succinate. On the other hand, pyruvate, fumarate and malate reduced the inhibition. Malonate also failed in the parasite brei to bring about an accumulation of succinate.

An enzyme which oxidizes specifically α-hydroxymonocarboxylic acids has been isolated from a wide variety of green plants [Clagett, Tolbert & Burriss (57)]. It could not be demonstrated in leaves of certain species or in etiolated leaves or embryos. Lactic acid apparently consumed 0.5 mole of oxygen and glycolic acid, 1.0 mole, the latter corresponding to production of oxalic acid, which is found in quantity in many plants. Because of the intensity of these oxidations, the authors suggest that the α-hydroxymonocarboxylic acids may act as hydrogen donors to reduce the quinone form of polyphenols which are apparently important in the terminal oxidations of many plants. Laties (58)shows that addition of glucose, pyruvate, citrate, or almost any member of the Krebs cycle restores a normal level of respiration following subjection of excised barley roots to fluorides. Acetate produced an immediate deleterious effect. Ceithaml & Vennesland (59) obtained a preparation from expressed and dialyzed parsley root, which contained oxalacetic carboxylase, malic dehydrogenase, oxalosuccinic carboxylase and isocitric dehydrogenase, but not aconitase or fumarase. According to Lynen & Lynen (60) in *Aspergillus niger*, citric acid is converted to oxalosuccinic, which is split to oxalic and succinic. Succinic is oxidized to oxaloacetic, which gives rise to oxalic plus acetic, the latter being converted to a second molecule of oxalic. Levine & Novak (61) found that all fatty acids from acetic through caprylic stimulated the oxygen uptake in *Blastomyces dermatitis;* longer chains inhibited. Increase in the number of double bonds, i.e., oleic, linoleic, had no effect on this inhibition.

Various Relationships

Carbohydrate.—Lifson, *et al.* (62) studied in detail the incorporation of acetate and butyrate into glycogen of previously fasted rats, fed glucose together with the specific acid. Acetate and butyrate labeled in the carboxyl and butyrate with C^{13} in the β-carbon gave rise to glucose labeled only in the 3- and 4-positions and therefore provided evidence that incorporation from the carboyxl carbon occurred only through carbon dioxide fixation. Both acids with C^{13} in the α-carbon gave rise to glycogen having the isotope in all positions of the glucose molecule, carbon atoms 1,6 and 2,5 having equal amounts, considerably greater than the amounts in atoms 3 and 4. They thus demonstrated that the entrance of the α-carbon of these two acids can occur by other than carbon dioxide fixation. The authors follow the chemical changes which may occur during repeated revolutions of the cycle and demonstrate that the pathways provided are capable of explaining all the results obtained in their experiments. In a further study, Gould, *et al.* (63) using carboxyl labeled acetate, found evidence that the two carbon atoms of acetate, in confirmation of the previous observations of Lorber, Lifson & Wood (64) were incorporated into the glycogen without previous combustion.

Sterols.—Little & Bloch (65) showed that cholesterol synthesized by liver slices in the presence of acetate with C^{14} in the carboxyl carbon con-

tained more isotope in the nuclear portion of the molecule than in the side chain, while that of the side chain equaled or exceeded the nuclear isotope when methyl-labeled acetate was employed. Chemical degradation of the side chain showed that the isopropyl group is derived from acetic acid. By employing doubly labeled acetate they demonstrated that some decarboxylation had occurred during synthesis.

The ready incorporation of the carbon of acetic acid into cholesterol has provided an additional method for the identification of substances which are metabolized like acetate. Borek & Rittenberg (66) found that rat liver slices, incubated with deuterioacetone, yield deuteriocholesterol. They also showed that isotopic acetone administered to rats together with α-amino-γ-phenyl butyric acid gave rise to the labeled acetylated amino acid. The authors could not decide whether acetone is first converted to acetate or is directly incorporated and later split. That acetone is not as readily available a precursor as acetate, would suggest the former, but as the authors point out, the physical properties and the physiological effects of acetone and acetate are so different that comparison on a simple basis is not possible.

Ottke, Tatum, & Simmonds (67) employed a mutant strain of *Neurospora* which required acetate for growth. From a comparison of the infra-red absorption spectrum of ergosterol elaborated in a medium containing deuterioacetate with that obtained in the presence of heavy water they concluded that deuterium from the acetate tends to appear on the more unsaturated carbon atoms of ergosterol while the carbon atoms from heavy water may appear at more saturated centers such as methyl and methylene groups.

Proteins.—Anfinsen, Beloff & Solomon (68) secured incorporation of the carboxyl carbon of acetate into the dicarboxylic amino acids of liver proteins of rats *in vitro*. They demonstrated that this incorporation was not due to combustion of the acetate to carbon dioxide and its subsequent fixation. The pathways of the synthetic reactions were not clear, however, since glutamic acid, aspartic acid, asparagine, glutamine, malate, pyruvate or alanine, added to the incubation medium failed to lower the activity of the C^{14} incorporated from acetate. In contrast, Lee & Lifson (69) recovered isotopic succinate from urines of rats administered isotopic acetate and non-isotopic malonate and found that the location of the isotope was in the carboxyl carbon of the succinate, corresponding to the distribution predicated from the reactions of the cycle.

Greenberg & Winnick (70) found the C^{14} of methyl- and carboxyl-labeled acetate in the intestinal proteins and liver proteins of rats 48 hr. after injection of 32 μc of C^{14} contained in 20 mg. of acetate. Glutamic acid, aspartic acid and alanine, isolated from the intestinal proteins, were radioactive, as was also arginine from the liver proteins. These four amino acids accounted for 97 and 87 per cent respectively of the activity of these two proteins.

Carbon dioxide fixation represented about 33 per cent of the total C^{14} in either protein, the remainder probably being incorporated without prior oxidation to carbon dioxide. The proteins of the intestinal mucosa contained

activity amounting to approximately 0.18 per cent of that of the bicarbonate administered, while they accounted for 1.75 per cent of the administered $C^{14}H_3COONa$ and 1.61 per cent of the $CH_3C^{14}OONa$.

Amino acids.—Sprinson (71) demonstrated that glycine labeled in the methylene group gives rise in the intact rat to acetic acid labeled in both carbon atoms. Isolated aspartic acid also contained the isotopic carbons in the α- and β-positions. The author offered two possible explanations. The first was that one glycine molecule upon deamination reacts with a second to produce a four-carbon α,β-labeled compound, which is in equilibrium with aspartic acid. Doubly labeled acetate would be derived from pyruvate as an intermediate. The second explanation proffered was that one molecule of glycine is degraded to a molecule of formic acid [Ratner, Nocito & Green (72)], which condenses with a second glycine molecule to give α,β-labeled serine as demonstrated by Sakami (73). Pyruvic acid, derived from serine would be the precursor of doubly labeled acetic and oxaloacetic acids, labeled in the α,β-positions [Chargaff & Sprinson (74)]. In addition to throwing light on the relationship between the two amino acids, glycine and serine, these experiments are of importance in demonstrating that a mechanism exists for synthesis of acetate in the body. The carboxyl carbon of glycine, like that of acetate, appears only in carbon atoms 3 and 4 of glucose [Sakami (75)], and hence probably is incorporated solely through the respiratory carbon dioxide. Siekevitz & Greenberg (76) have confirmed the reaction above for incorporation of glycine in serine. They call attention to the nonreversibility, under the conditions of their experiments, of the production of a one-carbon molecule from glycine.

Altman (77) found the methyl carbon of glycine labeled with C^{14} in the fatty acids synthesized in bone marrow homogenates from the femora, tibiae and humeri of rabbits. The saturated fatty acids contained the isotope in higher concentrations than did the unsaturated. Degradation of these fatty acids would have been of interest, because if the path of synthesis of the active two-carbon unit from glycine was similar to that proposed for acetate by Sprinson (71) the isotope should have been present in all the carbon atoms of the fatty acid chain.

Arnstein & Neuberger (78), starting with acetate labeled with C^{14} in the methyl and carboxyl groups, respectively, showed that acetate gives rise to glycine in rats. DL-aminophenylbutyric acid and sodium benzoate were fed simultaneously. The acetyl derivative of the amine was isolated and its activity measured. Hippuric acid was also isolated, hydrolyzed, and the glycine degraded and analyzed. The carboxyl carbon of acetate appeared exclusively in the carboxyl group of glycine, a distribution to be expected, as the authors point out, if acetate enters the tricarboxylic acid cycle by conjugation with oxalacetate, and after passage around the cycle, is converted to pyruvate and subsequently to serine by a reversal of the reaction postulated by Chargaff & Sprinson (74). Degradation of serine to glycine would take place by splitting off the β-carbon [Shemin (79)]. It may be ob-

served that these reactions are not the reverse of those postulated above for the production of acetate from glycine. We should have, in effect, the following series:

$$2\ C^{14}H_3\text{-}C^{13}OOH \rightarrow 2\ C^{14}H_2 \cdot NH_2\text{-}C^{13}OOH \rightarrow 1\ C^{14}H_3\text{-}C^{14}OOH.$$

Through a series of experiments in which leucine with radioactive carbon in the beta and gamma positions, respectively, was incubated in the presence of liver slices and administered subcutaneously to fasted, phlorhizinized rats, Gurin *et al.* (80, 81) elucidated some of the steps by which acetoacetate is formed. They concluded that the carboxyl carbon is first lost, then from the alpha and beta carbons a two-carbon unit is derived which condenses at random to give rise to ketone bodies. From β-labeled leucine, zero or low activities were associated with all the degradation fractions except that from acetone which included that derived from the residual carbon atoms of leucine plus the acetone formed in the chemical degradation of acetoacetate. Isovaleric acid is a probable intermediate, but not butyric acid.

According to Schepartz & Gurin (82), phenylalanine injected subcutaneously gives rise to acetoacetic acid in the phlorhizinized rat. Similar results were obtained with liver slices. When phenylalanine was labeled in both the carboxyl- and α-positions of the chain, the resulting acetoacetate was radioactive in the carboxyl carbon only. When labeling occurred in positions 1, 3, and 5 of the ring, the radioactivity of the acetoacetate was located in the terminal carbon. The authors therefore concluded: two carbons of the side chain, the α- and β-carbons, and two carbon atoms of the ring, carbon atoms 2 and either 3 or 1, take part in the formation of the acetoacetate. Migration of the side chain therefore must occur with liberation of a four-carbon residue which does not fragment and randomize.

Weinhouse & Millington (83) have reported in detail their preliminary communication demonstrating ketone body formation from tyrosine. Among their methods described is the especially valuable one in which, by a gentle permanganate oxidation, the carbon atoms in the different positions of acetoacetate can be identified. The authors showed that the β-carbon atom of tyrosine corresponds to the α-carbon of acetoacetate. As in phenylalanine (82) the ketone bodies apparently arise from an intact four-carbon unit, two carbons of which must originate from the side chain and two from the ring. A very low isotope concentration of the respiratory carbon dioxide indicates that tyrosine is not completely metabolized under these experimental conditions.

Metabolism of Unsaturated Fatty Acids

Development of chromatographic methods for the isolation of linoleic and arachidonic acids or their methyl esters [White & Brown (84), Riemenschneider, Herb & Nichols (85)] affords the prospect of more precise identification of these acids in metabolic experiments than has hitherto been possible. If these methods can be developed quantitatively, many problems, now difficult of solution, should become resolvable. Riekehoff, Holman &

Burr (86) point out some of the problems. They use standards of cod liver oil in lieu of absolute standards of the pentenoic and hexenoic acids which contribute to the absorption in the quantitative determination of unsaturated fatty acids by spectrophotometric methods. On account of the resulting high error Riekehoff *et al.* were unable to determine conclusively whether linoleic acid is retained in the tissues of the fat-starved rat. They were able to say that trienoic acid is present in relatively high amounts and that on supplementation of the fat-deficient diet with corn oil (containing linoleic acid) a deposition of arachidonic takes place. The polyunsaturated fatty acids are laid down primarily as phospholipids which may protect them from oxidation in the mitochondria, in which oxidation of the free linoleic acid occurs readily (36).

Barki *et al.* (87) studied the metabolism of polyunsaturated fatty acids in adult rats depleted by low food intake (5 gm. per rat per day) while on a fat-free diet and re-fed *ad libitum* for 35 days. Analyses of the fats before and after re-feedings were carried out by alkali isomerization and spectrophotometric analyses, [cf. (86)]. In both cases there was some uncertainty as to whether any linoleic acid was present during depletion. At this time, arachidonic acid also was low. Rats maintained on the same diet for an additional 36 days gradually lost the deficiency symptoms and a considerable increase in the levels of both linoleate and linolenate appeared. Supplementation of the diets with ethyl linoleate was accompanied by increase in the total fat and in its content of linoleate and arachidonate. The authors suggest that small amounts of the essential fatty acids may be synthesized in the mature rat.

Swern, Stutzman & Roe (88) propose the preparation of the amide derivatives of long chain unsaturated fatty acids for identification purposes. They prepared and characterized amides of oleic, elaidic, linoleic, ricinoleic and 10-hendecenoic acids. Many problems as to the preparation and isolation of these amides from mixtures would have to be solved before their usefulness in biological fluids could be demonstrated.

Lundberg, Chipault & Hendrickson (89) studied the mechanism of the autoxidation of methyl lineolate produced at room temperatures by blowing air through the fatty acid. From spectroscopic observations they suggest that the initial products are monomeric monohydroperoxides, mostly conjugated. Gradually the reactions become more complicated as further oxidations take place, and some of the products are identified. Franke, Lee & Siewerdt-Kibat (90) state that in microbiological experiments with acid fast bacteria, the oxidation of unsaturated fatty acid appears to start near the carboxyl group rather than at the double bond.

Fatty Acids and Growth

Continuing an interesting series of articles on the relation of the level of dietary fat to growth, Black, French & Swift (91) studied its relation to spontaneous voluntary activity in the mature rat. Activity was computed as

the difference between the total heat production measured with and without activity. The diets were so compounded as to be isocaloric, with the same levels of protein, vitamins and minerals in the daily allowances. The amounts fed were such as to maintain the rats in equilibrium or with a slightly positive balance. The authors measured the energy intake in food, output in excreta as well as respiratory exchange and found that the total heat production with activity unlimited was not appreciably affected by the level of fat intake; but with activity restricted heat production was markedly affected. Since the metabolizable energy of the food consumed was essentially the same on the two diets, they concluded that the difference during enforced inactivity must represent energy normally expended on activity, which must be greater on the high fat diet. Black *et al.* (92) measured the total heat production on supermaintenance and maintenance diets and heat increment (difference between the two) and demonstrated again that higher fat intake increases the efficiency of utilization of metabolizable energy. Swift & Black (93) review briefly the work of their group.

Pearson & Panzer (94), using essentially the same method failed to find increased weight in rats fed diets equalized with respect to protein and calories, but containing no fat (except for 30 mg. linoleic acid per day), and 8 per cent fat respectively. They did not determine the nonfat content of the carcasses at the close of the experiments. Their findings, that rats on the low fat diets excreted more of the essential amino acids, testifies to the greater efficiency of the diet containing fat and a lower protein content of the carcasses would probably have been found. Medes (95) attempted to replace the fat of the diet, except for supplements of linoleic acid, with various possible metabolic precursors, particularly aspartate, acetate and pyruvate, but found these compounds were unable to substitute for fat in stimulating growth of the albino rat.

Dunning, Curtis & Maun (96) found that restricting the fat of the diet to 25 calories daily per rat retarded the growth rate of diethylstilbestrol-induced mammary cancers. Experimental lymphosarcomas induced by Tedeschi (97) by injection of 1,2,5,6-dibenzanthracene into fat depots of mice were not influenced either as to incidence or rate of growth by differences in caloric intake. Bernhauer *et al.* (98, 99, 100) described conditions for cultivation of various molds with high and low fat content. Low nitrogen in the medium presumably limits growth, with the result that additional fat is synthesized and stored.

A few studies have been added to last year's contributions toward the elucidation of the role of biotin in fat metabolism. Trager (101), following up his earlier report on the replaceability of biotin by oleic acid for growth of microorganisms (102) demonstrated that either oleic acid, an oil from hydrolyzed plasma, or lecithin is capable of replacing biotin for growth of the yellow fever mosquito larva, *Aedes aegypti*. If biotin functions in the synthesis of fatty acids, it is difficult to understand how conversion of one lipid to another can occur without the vitamin, yet this must take place if

growth ensues from addition of oleic acid or lecithin alone. Evidence has increased that biotin functions through some complex-formation [Lichstein (103)]. Axelrod, Purvis & Hofmann (104) reached the same conclusion by the inhibition of the decarboxylation activity of yeast cells by structural analogues of biotin. Since the stimulatory effect of aspartic acid on yeast fermentation was not reduced by the structural analogues, the authors ascribed their effect to inhibition of a biosynthetic union of biotin or a biotin derivative with some other tissue constituent—possibly the specific protein. Insufficient evidence has been presented to decide whether the same biotin complex operates in carbon dioxide fixation as in oleic acid synthesis. Since coenzyme A has been shown to be essential for fatty acid synthesis, it should be considered whether biotin may function in the synthesis of this coenzyme.

Hodson (105) studied the cholineless mutant of *Neurospora crassa*, which he had suggested as suitable for biotin assay. He found that a combination of oleic acid and aspartic acid could not replace biotin for the mutant which differs from a number of lactobacilli. Oleic, vaccenic, linoleic and ricinoleic acids are able to replace biotin for growth of the anaerobe *Clostridium sporogenes* [Shull, Thoma & Peterson (106)], the more highly unsaturated acids being less effective. Some metabolic relationship is inferred between oleic and linoleic acids, the latter either being convertible directly to oleic, or giving rise to some common metabolic intermediate.

A particularly valuable study of the replaceability of oleic acid was reported in abstract form by Pollack, Howard & Boughton (107) using a diphtheroid bacterium, which could be cultured on a medium containing 18 amino acids, 16 growth factors and oleic acid, 0.5 to 10 μg. per ml. They studied the relative effects of a long series of acids. The *trans* isomers of oleic acid, elaidic, and petroselinic, 6-octadecenoic acid were active, but the 2-octadecenoic did not increase growth. Introduction of a hydroxyl group as in rincenoleic acid, 12-hydroxy-9-octadecenoic acid also failed to increase growth. All saturated fatty acids tested were inactive. Linoleic and linolenic acids could replace oleic, again indicating that they may be converted to oleic or metabolized through some common pathway.

Clostridium kluyveri is able to obtain its energy for growth from oxidation of four- and six-carbon fatty acids produced anaerobically from the two-carbon compounds, ethanol and acetate. Their metabolism may thus be studied, as pointed out by Stadtman & Barker (108) without complication of reactions involving utilization of carbohydrate. These investigators prepared a cell-free homogenate under standardized conditions, which catalyzes the reaction: Ethanol $\xrightarrow{-4H}$ "active" acetate $\xrightarrow{+\text{acetate}}$ four-carbon compound $\xrightarrow{+4H}$ butyrate. The active acetate is in approximate equilibrium with acetate. This series of reactions involves the transfer of four electrons during oxidation of ethanol to acetate and a second double pair of electrons during the reduction of the oxidized four-carbon compound to butyrate.

Since two electrons are believed to be transferred at a time, four intermediates are probably involved (109). Under appropriate conditions they identified acetaldehyde as the first reaction product and acetyl phosphate, formed from acetaldehyde and inorganic phosphate, as the second. The same enzyme preparations oxidized butyrate aerobically to a mixture of monoacetyl phosphate and acetate. Acetyl phosphate in catalytic amounts had to be present for oxidation to be initiated. The authors speculate on the possibility that monoacetyl phosphate may play the same role in bacterial oxidation of fatty acids that fumarate and adenylic acid play in corresponding animal enzyme systems. The oxidation of butyrate to acetate and monoacetyl phosphate could be reversed in an atmosphere of hydrogen by means of an active hydrogenase system (110) present in the homogenate. Acetoacetate, although of the same level of oxidation as the unidentified intermediate, is not the active product (111) since its reduction resulted in production of β-hydroxybutyrate with uptake of 1 mole of hydrogen and it was not further acted upon even in the presence of phosphorylating agents. β-Hydroxybutyrate, crotonate and isocrotonate are not the intermediates (112); vinyl acetate undergoes reactions which open the possibility of its acting as an intermediate, though not an obligatory one.

Summary,—The work of the year may be said to have been concerned predominantly with the preparation of more highly purified enzymes and with identification of cofactors in the biological oxidations involved in the Krebs cycle. As a result, contributions have been made toward the elucidation of the relation of the three-carbon unit of carbohydrate metabolism and the two-carbon unit characteristic of fatty acid metabolism to oxidations in the Krebs cycle and the conservation of the released energy through phosphorylations. With the development of methods of synthesis of isotopic amino acids and other complex compounds, mechanisms of their biological synthesis and degradation are being worked out, including their relation to these same units of carbohydrate and fatty acids.

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THE METABOLISM OF PROTEINS AND AMINO ACIDS[1]

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This review has been approached from the standpoint of the nutritionist. Protein metabolism, therefore, has been interpreted in terms (*a*) of the varied roles that proteins play in the maintenance of nutrition at various phases of the life cycle, (*b*) of the variability that exists among proteins as to their ability to support these several functions, (*c*) of the problems projected in attempts to elucidate the protein requirements of the animal organism in terms of constituent amino acids, (*d*) of emerging knowledge of the interplay of all nutrients in the food supply and the profound effect of the presence or absence of certain of these on the course of nitrogen utilization and metabolism, and finally (*e*) of the factors, extrinsic and intrinsic, that may alter the organism's disposition of its nitrogenous food supply.

The review does not cover all phases of the field or even any one phase completely. From the voluminous literature marking the achievements of the year, investigations have been chosen that indicate the trends that new information is taking.

The Nitrogen Requirement

Recent interpretations of protein needs in terms of constituent amino acids mark a milestone in the pathway of progress leading to an understanding of the many factors that affect the ability of an animal to utilize its nitrogenous food supply effectively. That the pattern of amino acids needed varies with species, the special demands of the various intervals of the life cycle, and the nutriture of the animal, is apparent. Very suggestive also is the idea that total over-all needs for various amino acids ultimately may represent a summation of the requirements of specific types of tissue (1). In addition, researches based on the administration of pure amino acids have provided information concerning types of synthetic reactions which can and cannot be accomplished and have exposed the many interrelationships—dietary and metabolic—that influence the course of protein metabolism.

For growth.—With the demonstration that growth may be achieved when highly purified amino acids replace protein in the diet of the rat (2), Rose created the base for a simple and trustworthy method for determining the nutritive significance of each of the recognized components of protein (3). Ten amino acids were found indispensable for growth in the rat and the exclusion from the food of any of these, other than arginine, led to profound nutritive failure, loss of weight, and eventual death. As a result, the amino

[1] This review covers the period from October, 1948 to October, 1949.

acids making up food proteins were classified as essential and nonessential in respect to their functions in nutrition. The improvement that has come with time in the simplified diets employed in tests of this nature has made it apparent, however, that these ten amino acids, when serving as the sole source of dietary nitrogen, are incapable of supporting growth at a rate commensurate with that induced by intact proteins and that some source of nonessential nitrogen is, indeed, essential (4, 5, 6).

Rose, Oesterling & Womack suggest (4) that the observed superiority of a dietary mixture containing all of the protein components to one carrying the ten essential amino acids only may mean that the synthesis of the nonessential amino acids with the concurrent elaboration of body tissue presents too great a burden upon the chemical resources of the cell. Syntheses do not, therefore, keep pace with the needs of the organism for optimum growth. It may be, also, that amino acids considered nonessential on the basis of studies in which low rates of growth were obtained may become limiting when growth proceeds at an accelerated pace.

The thought must be entertained, also, that certain of the nonessential amino acids are definitely indispensable to cell life and are not synthesized quickly enough to meet the demands of the labile metabolic pool. Such a condition can be conceived in respect to cystine, particularly in the light of Fischer's recent contribution (1).

The need of a supply of nonessential nitrogen in addition to the ten essential amino acids extends to other species, for example, the mouse (7). Growth on a ration containing 5.8 per cent of the physiologically available essential amino acids ranged from 43 to 51 per cent of that induced by an equivalent amount of casein, whereas that of a mixture containing 16 amino acids varied from 86 to 98 per cent. Brand & Bosshardt also have been able to sustain growth in mice by feeding a mixture of the L-amino acids prepared to simulate the amino acid composition of β-lactoglobulin, a pure crystalline protein of known amino acid composition which contains no prosthetic groups, phosphorus, growth-stimulating materials, or contaminating carbohydrate (8). In a third species, the dog, successful growth has followed the supplementation of a peptide-free basal ration with a peptide-free amino acid mixture containing the ten essential amino acids and about 35 per cent of nonessential amino acids (9).

Various sources of nitrogen may be used to provide the "necessary unessential" amino acids required in growth processes. It has been shown that dietary addition of single amino acids (4, 6, 7), mixtures containing only a few of the nonessential acids (6, 7), urea (10), or even ammonium salts (10, 11), may increase the growth-promoting properties of the essential amino acids for rodents. The rat has the capacity to use ammonium salts (10, 11); the mouse, however, is unable to utilize inorganic nitrogen fully (7). In general, however, it appears that the greatest growth occurs when several of the nonessential amino acids are represented in the test mixture (4, 6, 7).

It is of specific interest that certain of the nonessential amino acids ap-

pear to have growth-stimulatory properties. For example, evidences of enhanced growth in the rat caused by the dietary presence of tyrosine (6) and proline (12) have been reported. In the mouse, replacement of part of the glutamic acid supplementing a mixture of essential amino acids with DL-serine, DL-alanine, L-tyrosine, and DL-asparagine led to a significant increase in growth (7).

On the basis of observations like these, Sahyun has proposed that the classification of the protein-components be enlarged to include a third group to be known as "growth accelerators" (13). However, until more is learned regarding factors that may be involved in the utilization of amino acids, this group cannot be described in terms of specific amino acids. Noteworthy in this connection is the observation that stimulatory action may vary according to the general nutritional worth of the dietary nitrogen. Thus Rose, Oesterling & Womack (4) found glutamic acid stimulatory to rat growth when it supplemented a mixture of ten amino acids making up approximately 17 per cent of the ration. It did not, however, improve the nutritional value of one in which 18 other acids were present. Ramasarma, Henderson & Elvehjem (6) reported glutamic acid effective under certain conditions. The delineation, quantitatively and qualitatively, of the characteristics of a mixture of essential and nonessential amino acids capable of inducing growth as great as, or greater than that promoted by an intact protein, is one of the most interesting questions confronting nutritionists today.

Rates of growth induced by feeding amino acid mixtures to both the mouse and rat in most instances approximate, but do not quite equal, those characteristic of ingestion of intact protein (4, 6, 7, 206). This suggests that the requirements for growth of the various amino acids are not established yet on a quantitative basis. Unacceptability of the ration may be a factor (6, 7, 9). Ramasarma *et al.* have shown that equalization of food intakes by forced feeding resulted in a rate of growth in the amino acid-fed rat that was characteristic of the animal given casein (6). That failure to eat normally may represent a response to a dietary inadequacy (20) must not be ignored and, therefore, the reporting of food intakes in experiments involving the utilization of amino acids is important. The close correlation between efficiency in protein utilization and the energy value of the diet has become apparent. This and other factors possibly involved will be discussed in subsequent sections.

For maintenance.—An idea sown in the mind of a receptive student may reach full fruition decades later. Such indeed is the history of the researches beginning with the classic studies of Osborne & Mendel (14) on the nutritive rôle of the amino acids and culminating with the approximate establishment of the amino acid requirements of man by Rose (15, 16). Marked by a continuity of purpose and thoughtful and precise experimental execution, this series of investigations will stand long as a challenge and inspiration to research workers.

Today's achievement (16) depended upon the discovery, the identifica-

tion, and the demonstration of the essential nature of the amino acid, threonine (2). Studies were initiated with the adult human being in 1942, evaluations being made with nitrogen balance as the tool of measurement. Thus, lysine, leucine, isoleucine, methionine, phenylalanine, threonine, tryptophane, and valine were proved essential for the maintenance of body tissue (15). The exclusion of any of these amino acids from the diet was followed by "a pronounced negative balance, a profound failure of appetite, and a marked increase in nervous instability." A measurable loss of body protein occurred within 24 to 48 hr. after the omission of any one of these amino acids—an effect so immediate that one questions whether there can be measurable stores of the amino acids in the body. In passing from the qualitative to the quantitative aspects of the problem (16), Rose and his colleagues determined the quantity of each essential amino acid that was associated with the maintenance of positive nitrogen balance by progressively lowering the intake, a distinctly negative balance being induced at some stage of the test. The diets furnished approximately 10 gm. of nitrogen daily in most of the tests, glycine and urea supplying nitrogen for the synthesis of nonessential amino acids. Respective quantities of each of the eight amino acids needed for adult maintenance are presented in Table 1.

TABLE I

MINIMUM AND RECOMMENDED INTAKES FOR NORMAL MAN WHEN DIET FURNISHES SUFFICIENT NITROGEN FOR SYNTHESIS OF NONESSENTIALS*

(Strictly Tentative Values)

Amino Acid	Minimum Daily Requirement	Recommended Daily Intake	Subjects Tested
	gm.	*gm.*	*no.*
L-Tryptophane	0.25	0.5	31†
L-Phenylalanine	1.10	2.2	22
L-Lysine	0.80	1.6	27
L-Threonine	0.50	1.0	19
L-Valine	0.80	1.6	23
L-Methionine	1.10	2.2	12
L-Leucine	1.10	2.2	8
L-Isoleucine	0.70	1.4	8

* From Rose (16).

† All of these subjects have been kept in balance on 0.3 gm. or less.

It is of interest to note that Baldwin & Berg also estimated that 225 mg. of L-tryptophane per day was needed to maintain or to restore equilibrium in the human being when a basal diet low in this constituent was fed (17).

Records of the year 1949 also carry the most precise statement that has been reported to date regarding the nitrogenous requirements of the well-nourished adult rat [Cannon *et al.* (18, 19)]. Having established previously the qualitative needs for maintenance (20), Benditt and his co-workers proceeded to the quantitative phases of the problem and estimated the minimum amount of each amino acid necessary for the maintenance of nitrogen equilibrium and weight balance from curves relating graded amino acid intake to nitrogen balance and weight change. The data indicated that nine of the amino acids required for the support of growth in the rat were needed by the adult well-nourished animal. Arginine was not required. In all instances, the quantity of each amino acid needed for equilibrium was somewhat lower than that for maintenance of weight. A final test showed that a mixture of these nine acids fed in the proportions required in the individual balance tests sustained nitrogen equilibrium and integrity of hepatic tissue. These data are not in accord with previously reported estimates of Burroughs, Burroughs & Mitchell (21). An explanation of the variant results perhaps may be found in the items listed by Brush *et al.* (22). It should be noted, however, that Mitchell (23) does not believe that lysine is required for adult rodent nutrition, suggesting that the rat can synthesize lysine at a rate that provides enough of the essential for maintenance but not for growth. On the other hand, Neuberger & Webster (24) found that body weight could not be maintained unless lysine was provided.

Swanson and colleagues have shown that the well-nourished rat has not the capacity to synthesize the nonessential amino acids at a rate that will meet the needs of body maintenance as measured by nitrogen balance (25, 26).

For repletion.—Steffee *et al.* have set up the requirements for the repletion of body tissue in hypoproteinemic rats (27, 28). These requirements represented the minimal quantity of each essential amino acid which, in conjunction with the remainder of the standard ration, produced gains in weight equal to those induced by feeding the complete standard ration to another group of standard depleted animals. Excellent gain in weight accompanied the supplementation of the basal ration with these minimal quantities of amino acids. Also, with different amino acid intakes, changes in carcass protein directly paralleled gains in body weight. The authors indicated that while actual quantities used have no absolute value except under the experimental conditions imposed, the proportions of amino acids required in relation to each other may be of significance. The repleting rat, for example, required from two to five times the quantities of certain amino acids needed for maintenance. The requirements for leucine and lysine were the greatest, and these amino acids are found in high concentration in mammalian muscles.

In repletion, as in growth, rats have a limited capacity for synthesizing the nonessential amino acids from the essential ones (29, 30). Frost (30) found that mixtures containing 20 to 25 per cent of the total nitrogen

as nonessential amino acid nitrogen were efficient. Glycine, glutamic acid, arginine, or ammonium citrate could be utilized as a source of nitrogen in the synthesis of new body tissue. Urea was less efficient than glutamic acid, arginine, or ammonium citrate. Studies with amino acid mixtures patterned after casein did not seem to indicate a special nutritive role for any of the nonessential amino acids. Glutamic acid was capable of replacing all of the others.

For the growth of the cell.—Studies relating to the nutritional requirements of isolated cultures of animal cells growing *in vitro* in a medium containing clotted plasma and embryonic tissue extract represent a new approach to the problem of amino acid requirements that has been pursued actively by Fischer in Copenhagen (31). Whenever a dialyzed culture medium was used, death and disintegration of the cells occurred within 24 hr. This observation laid the basis for the determination of the characteristics of the vital dialyzable materials so that finally a simplified nutrient medium was formulated capable of supporting growth of both myoblasts and osteoblasts. Fischer's recent experiments (1, 32) on the relative importance of amino acids in the maintenance of the nutrition of pure strains of tissue cells *in vitro* are significant. He proceeded on the assumption that those amino acids whose withdrawal caused a depression of cell growth were the acids that were normally metabolized and could, therefore, be regarded as indispensable. He found that cystine occupied a key position and was the only amino acid whose absence from the complete amino acid mixture led to complete inhibition of growth. In a culture of myoblasts, cystine could not be replaced by methionine. In addition, glutamine, tryptophane, arginine, and a histidine-proline mixture fell into the category of indispensable amino acids. Fischer's data also indicated that the amino acid requirement of various types of tissue cells may not be the same; the growth of myoblasts, for example, was not affected by an absence of lysine from the amino acid mixture, whereas that of osteoblasts was depressed profoundly. Perhaps, as Fischer says, it will be possible in the future "to map the amino acid diet for the maintenance of other types of tissue cells." The potential value of experiments like these in understanding nutritional processes and in determining requirements is apparent. It is interesting that morphological changes in the cells occurred with the imposition of an amino acid deficiency that was manifest from the time the cells began to migrate into the medium. Thin atrophic cells that looked like glass splinters and containing many fat vacuoles were characteristic. The condition was reversible with the restoration of the specific amino acid to the medium.

Problems Related to Nitrogen Retention

Although advances in knowledge during the interval covered by the present review are beginning to define the nitrogenous needs of the animal organism, studies with simplified and purified sources of dietary nutrients are opening up a myriad of problems that show the complexity of the path-

ways of protein metabolism and the many factors that influence its course. It is not surprising, therefore, that those investigators interested in establishing quantitative needs in terms of constituent amino acids emphasize that their data are tentative, representing first approximations under the specific experimental conditions imposed (16, 20, 28). Problems raised by the recent work in the field of nutrition will be brought forward; certain of these are general in nature, others more specific.

Nitrogen balance as a measuring tool.—In the final analysis, the precise quantity of dietary amino acids that must be presented to the animal organism for the attainment of a sound nutriture must be measured in terms of the many and varied rôles played by proteins in nutrition. They must function in the synthesis of tissue in prenatal and postnatal history, in the maintenance of the integrity of body tissues, and in the elaboration of important functional proteins in the body. Also, they are needed for the manufacture of body secretions, both internal and external, for the fabrication of tissue enzymes and body metabolites, and for the laying down of tissue reserves upon which the body may draw in times of illness, stress, or emergency. They may serve as important detoxifying agents. That the measurement of the nitrogenous needs of the animal thus becomes a complex problem has been recognized by others (16, 33). Certainly they cannot be estimated on the basis of one or two, or even three, functions.

To what extent is nitrogen balance useful in meeting this problem in the mature animal? Does the classic interpretation of nitrogen equilibrium indicate that the healthy adult organism has resources for the maintenance of good nutrition in respect to all indices?

Swanson and her co-workers have attempted to evaluate the nutritional significance of increasingly positive nitrogen retentions in terms of minimal, adequate, and luxus requirements (34, 35, 36). On the basis of some 300 balance tests, they established curves relating balance to intake of nitrogen with dehydrated defatted whole egg proteins and lactalbumin serving as the respective sources of dietary nitrogen. With each protein, at a certain level of intake, the curve lost its straight line character and became curvilinear, and this raised the question as to which point on this curve satisfied the overall and total requirements for nitrogen. At equilibrium? At the point where the curve broke? Or when its slope no longer changed? Evaluations made by Mukhopadhay (36) in terms of hepatic nitrogen indicated that the quantity of nitrogen in the liver remained approximately constant in a range represented by a shift in nitrogen retention of 300 mg. per 300 gm. rat, brought about by progressively increasing the dietary protein (egg) from 3.2 to 10.0 per cent of the ration. With further protein enrichment of the diet, the nitrogen of the liver increased linearly. The nutriture of two key groups in the lactalbumin series was studied more intensively (34, 35), i.e., those fed the 3.5 and 14.0 per cent lactalbumin rations. Eighteen measurements that reflect protein nutrition were made and compared with a similar set describing the healthy stock rat. It was observed that the nitrogen

balance characteristic of the stock rats fell on the nitrogen balance curve of the lactalbumin-fed animals at the point where it became curvilinear. The animals given the lactalbumin food and maintained in a slightly positive nitrogen balance (24 mg.) seemed to differ from the stock controls only in respect to what might be called total labile protein. Serum proteins increased both on a relative and absolute basis and appeared to be maintained at the expense of hepatic protein. If the animals were penalized at all it was in respect to body reserves useful in meeting physiological emergencies. On the other hand, a change from the stock colony to the lactalbumin ration containing 14 per cent of protein increased the retention of nitrogen about 100 per cent. The volume of blood increased with a concomitant increase in all measured blood constituents. Total labile reserves were at the level of those in the stock rat, but the concentration of plasma proteins was excessively high. Mukhopadhay also noted an increase in blood volume at the time that increments in hepatic protein were observed.

Thus, even in apparently healthy animals, there is considerable growth in bodily stores of nitrogen in adult life and nitrogen equilibrium does not indicate a complete filling of these reservoirs. The requirement probably, therefore, should be expressed in a balance well over needs for establishment of nitrogen equilibrium. The data, however, suggest that high retentions of nitrogen may not be associated with the best nutritional state; the hypothesis might be ventured that the definitely augmented blood stream may be a predisposing factor in the precipitation of degenerative disease at some later period of life.

Rose (16) has pointed out that the needs for nitrogen equilibrium may be altered during disease, detoxication, reproduction, or lactation. Also, under all conditions, certain tissues are in a state of constant growth (37). In man there are changes in stature, in size of head, face, chest, hands, and feet. Other tissues, like the epidermis and epidermal structures, are growing continually.

That the adult requirement for nitrogen should be set in terms of at least a slightly positive balance is indicated by Mitchell's recent evaluation of the extent of "adult growth" (37). In a reexamination of data obtained in 1908 and relating to the nitrogen and phosphorus balances of 23 young men, he found positive nitrogen balances of surprisingly high order corresponding to over 6 gm. of protein per day. He and his co-workers have considered this factor in evaluating the protein requirements of college women (38, 39).

Physiological state.—Carbohydrates and fat can be stored in body depots; for protein no such reservoirs exist. Reserves are found instead in the labile cytoplasmic proteins making up the plasma and the hepatic and muscular tissues of the body. An animal with all of its reserves filled is, theoretically, in nitrogen equilibrium; additional quantities of dietary protein will not put it into positive balance. However, in individuals presumably healthy and consuming diets that can be classed as adequate, these reserves are filled to unequal degrees. In less healthy individuals, in turn, these stores are even of

smaller magnitude. The degree to which stores are filled may be judged by the quantity of urinary nitrogen excreted when the animal is given a protein-free diet, being high when reserves are high and low when reserves are low (40). The quantity of high quality protein that is needed to maintain nitrogen equilibrium is directly related to the physiological state of the organism. There is ample evidence in the literature indicating that the protein minimum for establishment of nitrogen equilibrium is greater in the well-nourished than in the protein-deficient animal (36, 41, 42). Recent experiments of Sprinson & Rittenberg (43) indicate that the protein nutrition of the animal affects its ability to utilize inorganic nitrogen. They observed that the administration of labeled ammonia to rats given a high protein ration resulted in a nearly quantitative excretion of the nitrogen, whereas rats maintained on very low protein diets used an appreciable fraction of the ammonia for protein synthesis. In studies relating to requirements and metabolic effects of amino acids, *in toto* or individually, preparation and standardization of test animals in respect to protein reserves become important. Variations in physiological state may well explain certain nonconformity of data from laboratory to laboratory (4, 6).

Imbalance of amino acids.—The quantity and proportions of the amino acids present in the dietary mixture exert a profound influence on growth. The influence of amino acid excess was indicated as early as 1942 by Earle, Smull & Victor (44). More recently, Ramasarma, Henderson & Elvehjem (6) and Russell & Taylor (5) have shown that an increase in the quantity of essential amino acids fed is accompanied by depressions in rates of growth in the rat. That the mouse may react to an imbalance of amino acids is suggested by Maddy & Elvehjem (7). Excess quantities of a specific amino acid likewise may be reflected in unsatisfactory response. For example, Brown & Allison (45) found in one instance that 4.8 per cent of DL-methionine in a casein diet caused rats to lose weight and pass into negative balance and to excrete more creatinine. In a second experiment (46), results were not as pronounced and the concomitant feeding of glycine alone, or of glycine plus arginine, increased the positivity of the nitrogen balance. On the basis of experiments with normal human beings, Schwimmer (47) concluded that DL-methionine not only prevented the sparing of nitrogen, but increased nitrogen loss. Excessive administration of glycine has been shown to be deleterious to the dog (48) and to the chick (49). Handler, Kamin & Harris (48) noted that large amounts of glycine were toxic and that the excretion of six essential amino acids was elevated markedly when glycine was infused intravenously.

Mixtures of amino acids formulated according to the distribution of the amino acids in specific biologically efficient proteins should yield interesting results in feeding trials (8). Evaluation in terms of the principle of proportionality pattern of essential amino acids recently proposed by Cannon and his co-workers (28, 50) should be productive also. That a mixture of essential amino acids in which each amino acid bears the same relation to tryptophane

as it does in animal muscle is indeed suggestive in formulating rations for growth and repletion, since elaboration of muscle mass represents one of the major tasks in the growth process.

Rôle of the D-*amino acids.*—One report (8) has come to the authors' attention in which mixtures of amino acids employed contained only the naturally-occurring forms of the various acids represented. That the final statement of the nitrogenous requirement and of the part played by the non-essential amino acids must await such experimentation is suggested by the response of rats and dogs to D- and L-forms of tryptophane (4, 51). Although it is assumed that the D-forms of amino acids in the diet, while nonutilizable for direct protein synthesis or maintenance, are nontoxic to the host, Albanese and co-workers (52) have presented preliminary evidence indicating an "untoward nutritional effect" of D-tryptophane. The role of the D-amino acids in nutrition awaits clarification. Recent reviews pertaining to their metabolism in mammals (53, 54, 55) and their utilization by microorganisms (56) supply suggestive material.

Peptide configurations.—There appear to be unidentified substances in dietary proteins, probably of peptide nature (57), that affect retention of nitrogen. Mice and rats failed to grow (58, 59) and doubly depleted dogs did not maintain body weight (60) when their dietary nitrogen was presented in the form of amino acids only. Several groups of investigators have studied, therefore, the rôle of preformed peptides in effecting the utilization of nitrogen and, in general, their findings indicate the relative unimportance of these substances. The rat, for example, apparently used hydrolysates of protein proved deficient in strepogenin as efficiently as intact protein both for growth and repletion (6, 61). Puppies also grew well on a ration deficient in peptides (9). Maddy & Elvehjem (7) found that the addition of proteins, either rich or poor in the factor (58), to an amino acid mixture containing the essential amino acids plus glycine and glutamic acid, did not affect growth of the mouse in proportion to their strepogenin content. The presence in the mixture of free serine, glycine, and glutamic acid seemed to be required for maximum growth. Perhaps the growth responses reported by Woolley with strepogenin concentrates resulted from the addition of these three acids, particularly since the tripeptide, serylglycylglutamic acid, possesses partial strepogenin activity for microorganisms (62). In view of the significance of amino acid imbalance, it is clear that the optimum requirement for each amino acid must be determined before the need for accessory peptide-containing compounds can be settled. From another angle comes Fischer's observation (1) that animal cells growing in a culture medium responded less favorably to an assortment of amino acids than to polypeptide-amino acid mixtures to which they were similar in composition.

The time factor.—Metabolic mechanisms involved in the processes of fabrication and maintenance of tissue proteins require the simultaneous availability of all the essential amino acids (63, 64). Delayed feeding of even one amino acid will decrease the utilization of the mixture from which it was

omitted at an earlier feeding (65, 66, 68). It is significant that two nutritionally-deficient proteins do not supplement each other unless they are fed together (69, 70). Thus, the potential value of ingested proteins may be less than laboratory analyses indicate because all essential amino acids apparently must not only be available for absorption but must be liberated during ingestion *in vivo* at rates permitting mutual supplementation. Melnick & Oser (71) report that in products "improved or impaired by heat processing and exhibiting no change in amino acid composition or degree of digestibility, rate of enzymic digestion is critical." For example, methionine in raw soy meal was absorbed so late during the gastrointestinal journey that it failed to supplement the remaining amino acids. As a result, inefficient utilization occurred.

On the other hand, Harte and his co-workers have observed that the rat seemed able to integrate its intake of a reasonably good protein offered alternately at high and low levels (67). Retardation in growth of the rat also followed the temporal separation of protein and nonprotein portions of the diet (72). Perhaps the separate feeding, so commonly practiced in bioassay, of nitrogen-carrying fractions of the diet, vitamins, etc., should be reevaluated in light of this observation.

The importance of the "time factor" needs consideration in human nutrition as is illustrated by the work of Leverton & Gram (73). Redistribution of an adequate daily supply of protein from the three meals of the day to lunch and dinner only placed their subjects in negative nitrogen balance. This study, if certain attenuating factors recognized by the authors can be excluded, may form the basis for a recommendation that distribution of kind and quality of proteins appearing in the human dietary must be considered as well as total daily amount in determining the protein requirement. There may be need to reconsider experimental data in the literature on which protein requirements are based.

Caloric intake.—Nitrogen retention is also a function of caloric intake, and failure to recognize this fact may account in part, at least, for variability in data relating not only to protein and amino acid needs but to the nutritional value of proteins. Retention of nitrogen, for example, decreased in the mouse (74, 75), the rat (74, 76, 77, 78), the dog (79), and man (80, 81, 82) if the energy value of the ration was decreased. In the growing animal such decrements representing losses in body nitrogen leave a smaller proportion of absorbed nitrogen for the synthesis of new tissue; in the adult animal they are associated with loss of body substance. There is a critical point at which the metabolic break occurs. It seems that in dogs the lowest level to which the energy value of a ration can be reduced without affecting efficiency of utilization of food protein is approximately 50 per cent of the normal caloric intake (83). The critical point may vary with species (74). In hypoproteinemic rats (84), a caloric intake of 1,240 kcal. per sq. m. per day is essential for the regeneration of body tissue, a value coinciding with the energy requirement for the synthesis of new tissue (74). Also the catabolism of rats brought to a

constant plane of nitrogenous metabolism by the administration of a protein-free diet is not increased until the energy value of the ration is reduced by one-half (76, 77, 78). Bosshardt and co-workers have shown (75) that an increase in the caloric consumption at a constant protein intake improved protein utilization, which could be enhanced still further by the addition of calories in the form of added protein. The authors interpret this observation as meaning that in many states of semi-starvation, protein is the most important limiting factor. Beattie, Herbert & Bell (85) found that in emaciated individuals nitrogen was stored when the diet provided 35 Calories and 0.17 gm. of nitrogen per kg. of body weight. The absolute amount of nitrogen retained apparently was related directly to the caloric intake, provided the nitrogen intake was above the critical level for nitrogenous equilibrium.

An investigation has been reported by Anderson & Nassett (86), an extension of which will be awaited with considerable interest. A definite increase in the cost of metabolism occurred when the quantity of isoleucine was reduced to one-third. The specific dynamic action was elevated from 2.9 to 6.0 Calories per day per rat. On the other hand, a decrease in the daily supply of methionine lowered the efficacy of utilization of the amino acid mixture with no change in specific dynamic action. An increase in the daily caloric intake and allowance for adjustment to the various dietary regimes (87) may alter the outcome of the experiment.

The relation of the nitrogen requirement to the energy value of the diet is emphasized by Rose (16). More energy was needed to keep a subject in positive nitrogen balance when he consumed an amino acid mixture or an acid-hydrolyzed protein than when an equivalent amount of intact protein was eaten. Rose, therefore, followed the general practice of administering 55 Calories per kg. of body weight per day. He could give no categorical reason for these observations. The data indicate that the recommendations for the requirements of the human being for the essential amino acids are tentative. It should be noted that the experience of others in the feeding of low protein diets has led to the use of relatively high caloric intakes (38, 88).

Hegsted & Haffenreffer (89) write that the voluntary food intake of an animal is governed by means yet unknown at a certain percentage above its needs for normal basal metabolism. Gain will result to the extent that the protein, vitamin, and mineral needs are met by this amount of food. The remainder must be used presumably in activity. In view of this statement, the administration of a high caloric diet with an ineffective amino acid mixture might be reflected in extreme activity. Kuehl (25) and Clark (26) raise the question as to whether the hyperexcitability of adult rats fed a diet providing 60 Calories per day and a mixture of the ten essential amino acids only as their dietary nitrogen might not well be explained in terms of caloric abundance rather than nitrogen deficit.

Rôle of dietary fat.—The course of protein metabolism may be altered profoundly by the character of the nonprotein portion of the ration. The classic experiments on the relative protein-sparing effects of carbohydrates

and fats have been summarized by Deuel (90). Bosshardt and co-workers (75) have recently studied the influence of fat and carbohydrate calories on the utilization of proteins by mice receiving caloric intakes below the minimal level for optimum nitrogen retention. When all diets contained some fat, carbohydrate, and protein, fat and carbohydrate were equal in their protein-sparing effect. Allison, Anderson & Seeley (83) measured the decrease in nitrogen balance index induced by restricting the caloric intake of dogs. The addition of either fat or carbohydrate calories increased the nitrogen balance index to the same extent.

Despite observations of this kind, evidence is growing that fat exerts a rôle over and beyond the provision of calories and the essential fatty acids, and that this function relates to the control of protein metabolism (91 to 95).

Further evidence of the importance of fat in the regulation of nitrogen metabolism is found in the studies of Swanson and her collaborators in which the nitrogen metabolism of rats living on a protein-free diet was measured (76, 78, 96, 97). They observed that the catabolism of rats ingesting isocaloric quantities of high fat and low fat rations proceeded at essentially the same rate. However, upon the adjustment of the energy value of the diet to one-fourth of the customary caloric consumption, the elimination of fat doubled the destruction of body tissue. Supplementation of the low fat ration with 4 mg. of methionine nitrogen per day immediately reduced the catabolic processes, the nitrogen balance becoming of the same order of negativity as that characteristic of the animals receiving fat in their rations at this level of caloric intake. The effect of the elimination of fat has been confirmed in a series of four experiments conducted during the last three years.

Whereas no disturbances over and beyond those induced by the feeding of the high-fat low-nitrogen diet were observed in the rats offered the low fat ration *ad libitum* in the early tests, the more recent experiments (97) have indicated that profound metabolic disorders characterized these animals. When the caloric intake was maintained at 56 Calories per day by force-feeding, only 60 to 70 per cent of the rats survived the 30-day feeding test. The concentration of the amino nitrogen in the blood of the survivors was nearly double that of the fat-fed controls, while that of urea was abnormally low. The elimination of fats decreased the riboflavin content of the liver. The addition of methionine to the ration restored these values to levels characteristic of the animals receiving the high fat ration.

That fat is important in the diet of man also was demonstrated by Schwimmer and his co-workers (81, 82). The incorporation of 30 per cent of fat in a diet providing 900 Calories and 6.0 gm. of nitrogen daily decreased the urinary excretion of nitrogen of their subjects below that occurring on diets providing 10 and 20 per cent of fat. They believed that "the nitrogen sparing effect of 30 per cent fat was not due to increased calories, but rather to something intrinsic in the higher fat intake *per se*." Related to these

observations are those of Pearson & Panzer (92) showing that rats given a diet containing corn oil excreted significantly smaller quantities of valine, lysine, and methionine in the urine than rats fed a low fat ration.

Vitamin-amino acid relations.—The metabolic interdependence of amino acids and vitamins must be considered in evaluating the nitrogenous needs of the animal organism. Since this topic undoubtedly will be treated more fully in another review, the present discussion is restricted to a few current developments which highlight this relationship.

Amino acids may serve as precursors of certain vitamins, as has been exemplified so strikingly by the transformation of tryptophane to nicotinic acid (98 to 102). Only recently has it become possible to associate individual vitamins with such fundamental phases of over-all protein metabolism as deamination (103), transamination (104), transmethylation (105), oxidation (106, 107), decarboxylation (108, 109), and carbon dioxide fixation (110). Of such significance are vitamins in reactions of this nature that when a vitamin is required for the intermediary metabolism of an amino acid, that function may have the priority and the vitamin may be unable to serve also for reactions involved in other physiological processes (90).

Availability of amino acids.—Comprehensive studies of the distribution of amino acids in meat products (111, 112, 113) exemplify the effort that is being made to describe food proteins in terms of their constituent amino acids. Such studies will become important in defining the nitrogenous requirements of animals and man. The recent demonstrations of the reliability of the microbiological technique for estimating amino acids will make the information all the more valuable (114, 115, 116).

Recent investigations, however, have indicated that amino acid composition cannot be taken unqualifiedly as a criterion of protein efficiency. Just as important as the quantity of nitrogen and the content of the essential amino acids is the physiological availability of the protein units. For example, Kuiken & Lyman (117), by differentiating fecal nitrogen in terms of the 10 essential amino acids, have shown that the amino acids of roast beef are completely available, whereas those of cottonseed flour are not.

Technological and home practices in the handling of food may affect the nutritive value of proteins by destroying certain amino acids (118, 119) and by rendering the protein resistant to enzymatic attack during the digestive process. Evans & Butts (120) postulate that the linkages formed by the interaction of free amino and carboxyl groups or by the combination of protein-bound methionine, cystine, or histidine with carbohydrate moieties are enzyme-resistant. That the presence of carbohydrate in a processed natural protein definitely may influence the availability of the constituent amino acids is indicated (121 to 124).

Paradoxically, it often happens that decreased nutritive value in a processed product is not associated with a change in nitrogen content, in amino acid composition, or in digestibility (125). Instead it is related to a retardation induced by the processing procedure, in the rate of release of

individual amino acids in the intestinal tract, lysine being the limiting amino acid [Melnick & Oser (71)]. On the other hand, in those raw foods improved by heat processing, available methionine is limited in the raw product, and heat treatment increases the rate at which it may be liberated from the protein during digestion with resultant improvement in the overall nutritive value of the protein. Perhaps there is a definite pattern of liberation that gives optimal utilization of the essential amino acids. At any rate, the kind, the amount, and the rate at which amino acids are supplied to the animal body may have an important bearing on its total nutrition.

Pattern of amino acid excretion.—The excretion of many of the amino acids in the urine is increased when proteins of poor quality are fed (126, 127) suggesting that the pattern of urinary amino acids may serve as a tool in evaluating the quality of the diet consumed. Some attention has been given to the description of the normal pattern. Available data depicting the quantity of the ten essential amino acids excreted by subjects maintaining body weight and receiving adequate protein are in fair agreement (128 to 132). Eckhardt & Davidson (133) found that large decreases in the quantity of protein ingested resulted in only moderate decreases in the quantity of amino acids and peptides present in the urine, a finding corroborated by Steele *et al.* (134) who noted, however, that the concentration of certain urinary amino acids bore a direct relation to the level of dietary protein. When a diet adequate in calories but deficient in protein is offered, amino acids in the urine increase (133). This excretion "must be considered endogenous in origin." This factor, as well as the existence of individual patterns of amino acid excretion (135), should be recognized in studies of this nature. Wastage of amino acids in the urine associated with deficiencies of essential amino acids is not a characteristic of general weight loss, i.e., caloric, riboflavin, or thiamine deficiency [Sauberlich & Baumann (136)].

Intermediary Metabolism

General.—The development of new tools useful in charting the course of protein metabolism has stimulated interest in the mechanisms of biological syntheses. Studies by Borsook and co-workers (137, 138) and others (139, 140), indicate that every tissue thus far tested can take up, *in vitro*, into its proteins every labeled amino acid presented to it. Embryonic and malignant tissues and bone marrow cells are considerably more active in this respect than normal adult tissues (137, 140, 141). With the single exception of lysine, the uptake of amino acids depends directly upon respiration (137, 138), and is most efficient when the concentrations of the individual amino acids in the medium are in the physiological range. The labelled amino acids evidently are taken up in peptide bonds (137). Furthermore, a synergism may exist between two types of intracellular particles, i.e., microsomes and mitochondria. Rates of uptake of amino acids *in vitro* closely simulate rates observed *in vivo*. Lysine, glycine, and leucine are incorporated independently and at different rates (137, 138). Sanadi & Greenberg (142)

have shown that *in vivo* a deficiency of a single amino acid definitely retards the rate of incorporation of labeled amino acids in tissue proteins.

By measuring the rate of interaction of isotopic glycine with the body proteins in rats and human subjects, Sprinson & Rittenberg (143) estimated the rate of protein synthesis and the size of the nitrogen pool. The two species are very different in their rate of protein rejuvenation; the instability of cellular proteins, that gave rise to the concept of a dynamic state, is more typical of the rat than of man. The proteins of the internal organs apparently are involved more extensively in protein turnover than are muscle proteins, a large fraction of the latter being relatively inert metabolically.

The use of mutant strains of microorganisms has clarified many of the biochemical steps that mark intermediary metabolism and the biosynthesis of amino acids. The existence, in many instances, of a biochemical unity between higher and lower organisms lends importance to information thus obtained [Tatum (144)].

Knowledge is accumulating regarding the metabolism of the individual amino acids. The potentialities of a practical application of certain aspects of this information in solving the problems of dietary requirements in respect to certain nutrients are apparent in the following sections.

Aspartic and glutamic acids.—The central position of aspartic acid in amino acid metabolism is well recognized. Wu & Rittenberg (145) have indicated that the amino group of L-aspartic acid behaves metabolically like ammonia in giving rise to urinary ammonia and urea in the rat. The ratio of the concentration of N^{15} in urinary ammonia to that in urea was of the order obtained with ammonium citrate and considerably lower than with other natural amino acids. Anfinsen, Beloff & Solomon (146) studied the rôle of carbon dioxide as a building block in the synthesis of the dicarboxylic acids. They, as well as Greenberg & Winnick (147), found that the carboxyl carbon of acetate was incorporated without first becoming carbon dioxide; a major portion of the tagged carbon was located in the dicarboxylic amino acid fraction.

Glutamic acid participates extensively in transamination and in the synthesis of urea (148). Waelsch (149) now has postulated that the glutamic acid-glutamine system, by ensuring adequate reserves of ketoglutaric acid, may aid in regulating the concentration of metabolites entering the tricarboxylic acid cycle from various pathways. Mayer-Gross & Walker (150) found that parenteral administration of either glycine, *p*-aminobenzoic acid, or glutamic acid restored hypoglycemic patients in insulin coma to consciousness at a lower blood sugar level than did glucose.

Glycine and serine.—Although designated as a dispensable amino acid, glycine performs so many diverse and vital rôles in the physiological economy that it truly might be considered an "essential" amino acid. In addition to its importance in detoxification and in the synthesis of glycocholic acid and glutathione, glycine furnishes the sarcosine moiety of creatine, is readily

converted to ethanolamine which may be used in the synthesis of choline, and (151) enters into the structure of purines.

Its α-carbon atom is utilized in the synthesis of hemin (152), whereas the carboxyl carbon is found only in globin (153). The nitrogen atom is incorporated in all four pyrrole structures of protoporphrin (154, 155). The fabrication of these physiological units is accomplished swiftly (156), the bone marrow (157) being a site of synthesis.

The interconversion of glycine and serine in rat liver is extremely rapid and extensive (158). The hypothesis of Siekevitz & Greenberg (159) that formate derived from one molecule of glycine condenses with a second intact glycine molecule is in harmony with earlier observations of Shemin (160) and of Sakami (161), who recognized the methyl group of choline as a second potential source of formate in the synthesis of serine (162). The utilization of glycine in the synthesis of acetic and aspartic acids (163), of glycogen (164), and of fatty acids (165) has been demonstrated with methylene-labeled glycine.

Handler *et al.* (48) in studying the metabolism of glycine, have conducted a comprehensive investigation of the influence of continuous intravenous injection of the amino acid in the dog on the concentration of glycine and glucose in the blood, on the deposition of glycogen in the liver, and on the excretion of urea and of certain amino acids. *In vivo* studies of this nature coupled with *in vitro* experiments of the type conducted in the laboratory of Christenson *et al.* (166) will broaden present concepts of the routes taken by amino acids in the metabolic turnover.

Lysine.—The anomalous behavior of lysine in respect to reamination and to the action of tissue enzymes has been clarified further by Borsook and his colleagues (167, 168). In liver homogenates, the ϵ-amino group is first removed to yield α-aminoadipic acid which is oxidatively deaminated to α-ketoadipic acid from which α-ketoglutaric acid arises by oxidative decarboxylation. The conversion of lysine to α-aminoadipic acid before it yields its α-amino nitrogen thus explains its failure to participate in reversible transamination reactions *in vivo*. Although it is a precursor of lysine in *Neurospora* (169), α-aminoadipic acid cannot be utilized by the growing rat or by certain bacteria (170).

Methionine.—Considerable interest has centered on the manner in which the methyl radical attached to sulfur or nitrogen in certain important biological compounds enters into intermediary metabolism. Apparently it is transferred as a unit from methionine for the synthesis of choline and creatine without any exchange of hydrogen atoms (171). The methyl group of methionine participates actively in reactions involving both oxidation and transmethylation. Du Vigneaud and his co-workers (172) observed that in the intact rat, one-fourth of the administered methyl carbon appeared in the expired carbon dioxide during the first day, and approximately one-half of the tagged element was excreted in urine, feces, or carbon dioxide in a two-day interval. Transmethylation to form choline and creatine was a more

active process than incorporation of methyl groups into total body protein, although the presence of C^{14} in all major organs and tissues reflected the widespread participation of this essential unit in biological syntheses.

To the substances heretofore recognized as potential sources of the methyl radical, i.e., choline with its derivatives and betaine, now may be added dimethylthetin and dimethyl-β-propiothetin (105, 173). These methylsulfonium compounds are clearly marked as a new class of methyl donors effective in their own right with no dependence upon structural analogy to betaine for their activity (105). The effect of structural alteration is marked; dimethylthetin is both lipotropic and growth-promoting, whereas diethylthetin is ineffective.

Dubnoff & Borsook (173) have shown that dimethylthetin also may serve as an active methyl donor for homocysteine *in vitro*, and have isolated from liver and kidney an enzyme specifically catalyzing the process. The observations from the New York and California laboratories strongly suggest that dimethylthetin, a homologue, or a closely related derivative may represent a tissue constituent intimately involved in the methylation process. The concept of a pool of labile methyl groups has been modified by Dubnoff & Borsook (173) who propose that specific methyl donors exist for each methyl acceptor and that the physiological transfer of a methyl group from methionine to choline may represent a cyclic process in which some, and possibly all, steps are irreversible. An increased requirement for methyl groups or retarded demethylation of methionine may account for antilipotropic action when large amounts of cystine are fed to growing rats [Treadwell (174)].

The observation of Rutman, Dempster & Tarver (175) that genetic differences exist in the quantity of methionine incorporated in surviving liver slices taken from two strains of rats has important implications experimentally.

Leucine.—One pathway of leucine catabolism has been indicated by Coon & Gurin (176). The α- and β-carbon atoms appear to split off as a two-carbon intermediate which condenses with a similar unit to yield acetoacetate. Apparently the isopropyl group does not contribute to acetoacetate formation.

Phenylalanine, tyrosine, and dihydroxyphenylalanine.—Schepartz & Gurin (177) have investigated the oxidation of phenylalanine to acetoacetic acid with isotopes. They believe that it arises from a four-carbon precursor composed of two atoms from the side-chain and two from adjacent positions in the ring, and not by random condensation of two-carbon fragments. The distribution of the labeled atoms indicated a migration of the side-chain from its original position on the ring.

Apparently, a complex metabolic relation exists between tyrosine, ascorbic acid, and pteroylglutamic acid. It is known that ascorbic acid prevents the appearance of abnormal metabolites in the urine of guinea pigs following the addition of tyrosine to scorbutogenic diets. Woodruff *et al.* (178) have shown that pteroylglutamic acid also prevents hydroxyphenyluria in guinea pigs under similar conditions. It appears that pteroylglutamic acid, like

ascorbic acid, is an essential component of an enzyme system involved in tyrosine oxidation. It will be interesting to learn whether these vitamins act independently at different stages or participate simultaneously in a single metabolic step.

When either succinylsulfathiazole or 4-aminopteroylglutamic acid is administered to rats, homogenates prepared from the livers fail to oxidize tyrosine at a normal rate (107). Tyrosine oxidation, however, proceeds normally when the antagonist is added directly to liver homogenates from normal animals.

Ascorbic acid is required also for the metabolism of 3,4-dihydroxyphenylalanine. In 1947, Sealock & Lan (179) observed that kidney slices from scorbutic guinea pigs were almost totally incapable of oxidizing the amino acid unless ascorbic acid was added. Clegg & Sealock (180) now have reported that extracts of kidney from scorbutic and normal guinea pigs metabolize dihydroxyphenylalanine equally well. They suggest that the difference in activity of cellular and cell-free extracts reveals the highly complex rôle occupied by ascorbic acid in metabolism. In addition to the well known reactions of decarboxylation and deamination, they describe a new reaction involving the disappearance of the catechol nucleus of 3,4-dihydroxyphenylalanine.

Tryptophane.—Krehl (98) has carefully reviewed the metabolic relationships between tryptophane and niacin. The conversion of tryptophane to nicotinic acid seems to proceed similarly in mammals (101, 181) and in *Neurospora* (182). Kynurenine is first formed by oxidation of the indole ring of tryptophane, and 3-hydroxyanthranilic acid is a probable intermediate between kynurenine and nicotinic acid. Kynurenic acid does not participate in nicotinic acid synthesis since the side-chain of the amino acid is apparently lost (181, 183). The alleviation of pellagrous lesions (99), and the enhancement of tissue stores of nicotinic acid in man (100) and in the rat (102) following the administration of tryptophane emphasize the significant rôle played by tryptophane not only as an essential structural unit, but also as the precursor of a vitamin required for the normal activity of certain enzyme systems. In man, as in other species, the excretion of xanthurenic acid after a test dose of tryptophane is one of the early signs of pyridoxine deficiency (184).

That kynurenine may also be converted to alanine and anthranilic acid has been reported by Wiss & Hatz (185) who noted an increased concentration of alanine in the livers of young rats fed either L-tryptophane or DL-kynurenine. Wiss (186) later obtained an acetone-dried extract of the kynurenine-splitting enzyme which attacks only the L-isomer.

Structural analogues.—It is not clear whether the swift, profound interference with metabolic processes that characterizes the action of certain antimetabolites is due to the blocking of an enzyme system, the incorporation of a physiologically useless unit in the protein matrix, or to unknown mechanisms.

Dietary ethionine retards growth in young rats (187, 188, 189). Tarver

and his group have studied its action in protein synthesis and believe that it exerts an inhibitory effect interfering with the incorporation of methionine and glycine in liver proteins (190), with the transfer of sulfur from methionine to cystine (190), and with normal deposition of fat in the liver (191). It accelerates the catabolism of body tissue in rats partially depleted of their protein reserves and fed a nitrogen-low ration (192, 193). Total urinary nitrogen is greatly increased, the increment being due to "undetermined nitrogen" and not to urea. Methionine sulfone acts similarly (193), an observation in accord with that of Bennett (194) who noted the adverse effect of the methionine derivative on growing rats.

A potent antagonist of phenylalanine in microorganisms (195) and in the rat (195) is β-2-thienylalanine, its action being reversed by phenylalanine (196, 197). The amidation of glutamic acid may be blocked by β-hydroxyglutamic acid (198), an inhibition counteracted either by small amounts of glutamine or large amounts of glutamic acid.

The biosynthesis of urea.—One of the significant developments of the year has been the expansion of the ornithine cycle and its direct linking with the tricarboxylic acid cycle. The varied aspects of the problem have been summarized by Ratner (148, 199, 200), who has proposed a scheme that apparently represents a major pathway of urea formation. Aspartic acid and citrulline are converted anaerobically to arginine and malic acid, adenosinetriphosphate being directly involved in the reaction. The ureido carbon for the synthesis of citrulline from ornithine is furnished by carbamyl glutamate. The tricarboxylic acid and ornithine cycles are connected by transaminase and by two simpler cycles, one of which permits the catalytic turnover of α-ketoglutaric acid as a carrier of ammonia by means of glutamic dehydrogenase; the other permits malic acid formed in arginine synthesis to function catalytically in aspartic acid formation by means of malic dehydrogenase.

Biological Evaluation of Proteins

Allison, in two recent reviews (41, 201), has covered thoroughly the basic concepts of protein metabolism that must be considered in the estimation of the nutritive quality of the various food proteins. Evaluation may be made through nitrogen balance, growth, tissue regeneration, and amino acid analysis. Obviously, the biological indices measure different functions. Even in one type of study, tissue regeneration for example, the ability of a single dietary protein may vary in supporting the elaboration of different functional proteins in the body. Thus (40), the proteins of egg favor the production of plasma proteins, whereas those in beef muscle permit the synthesis of three to four times as much hemoglobin as plasma protein. Wheat gluten, generally considered an inferior protein, favors the formation of plasma proteins more than do egg proteins or casein. It is obvious that real understanding of protein needs will come when nutritive values of the many food proteins have been assessed in terms of specific physiological effects. A start toward this end has been made. In a collaborative study sponsored by Rutgers University, six reference proteins are being tested in various laboratories. Results

of the assays are beginning to appear in the literature (40, 61, 202 to 205), but the data will become more significant when they are evaluated as a unit.

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CHEMISTRY OF THE HORMONES[1]

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The scope of this review is restricted to the chemistry of the nonsteroid hormones, inasmuch as the chemistry of the steroid hormones is presented elsewhere in this volume. Moreover, the rather comprehensive review of hormone chemistry by Wettstein & Benz (1) in the previous volume of the *Annual Review of Biochemistry* has made it possible to limit this chapter almost entirely to publications which have appeared within the last 18 months. References to earlier work will be made only for the sake of continuity of the presentation.

Insulin

Interest in the chemistry of insulin has continued particularly in the application of the newer techniques of protein analysis in order to obtain more information regarding the structure of this protein hormone. The papers reviewed here have been selected from those which have appeared since the review by Wettstein & Benz (1), which devoted a good deal of attention to the more recent work on the chemistry of insulin.

The behavior of insulin in the ultracentrifuge has been studied in two laboratories. Gutfreund (2) confirmed his previous observations (3), based on osmotic pressure measurements, that insulin in solutions of pH 7.0 to 7.5 and a concentration of 0.4 to 0.9 per cent was homogeneous from the point of view of particle size, and had a maximum molecular weight of 47,000 to 48,000. However, at pH above 7.5 or below 4.0, or in more dilute solutions, evidence was obtained for reversible dissociation of the protein hormone. The optimum pH for dissociation was between 2 and 3. Under the influence of the combined effect of pH, dilution and temperature, the minimum molecular weight from osmotic pressure determinations was $12,000 \pm 500$. Similar data ($12,000 \pm 530$) with respect to minimum molecular weight were obtained by calculations based on amino acid analysis. It was concluded that insulin consists of subunits with a molecular weight of 12,000 and that the forces holding these subunits together involve the ionizing groups of the protein. Observations of this dissociation phenomenon by osmotic pressure and ultracentrifugal methods are in good agreement. Oncley & Ellenbogen (4), using the ultracentrifuge, studied the molecular state of insulin as a function of the insulin concentration, the pH, and the ionic strength of the solution in order to examine the forces involved in the polymerization of the insulin subunit of molecular weight 12,000. The data showed that the sedimentation constant was a function of the net charge of the insulin and the ionic strength of the solution. The monomer of molecular weight 12,000 had a maximum net

[1] Miss Annette Terzian gave invaluable assistance in the assembling of the literature upon which this review is based.

charge of +12 at pH 2 or less, and in 10 *M* sodium chloride solution, a sedimentation constant of about 1.9 S. (Svedberg Units) was observed at this charge. As the net charge was decreased or the ionic strength increased, the sedimentation constant increased to 3.5 S. All the measurements made, under conditions where insulin monomer predominated, agreed well with the view of a rather symmetrical monomer molecule of a molecular weight of 12,000. In the pH range from 7 to 8, the state of polymerization of insulin appeared less clearly defined; the evidence suggested that neither trimer nor tetramer molecules completely satisfied the observed measurements. In the case of insulin sulfate crystals, it has been possible to demonstrate that the molecular weight of the insulin in the crystals is approximately 12,000, in agreement with x-ray diffraction measurements. The existence of insulin as a monomer of molecular weight 12,000 would aid in visualizing the passage of this protein hormone into cells.

On the basis of solubility measurements, electrophoresis, and equilibrium dialysis, Fredericq & Neurath (5) obtained evidence that insulin binds reversibly with thiocyanate ions. Equilibrium dialysis measurements at pH 3 to 4 did not show any clear maximum value for the amount of bound thiocyanate ions. These results appeared to be independent of the zinc content of insulin. Electrophoretic measurements in the low pH region showed a marked decrease in the net positive charge of insulin in the presence of thiocyanate; the homogeneity of the protein remained unaltered. Ultracentrifugal analysis between pH 1.5 and 3.0 revealed that increasing concentration of thiocyanate promoted the association of insulin subunits. The pH range of association was not significantly affected by thiocyanate, but the mean molecular weight of insulin increased in proportion to the concentration of the thiocyanate ion.

Velick & Ronzoni (6) presented a complete analysis for the amino acid composition of insulin. The data are in good agreement with previous data (7), with the exception of serine and threonine. The present investigators find values of 6.6 per cent and 3.5 per cent respectively for serine and threonine, as compared to values in the literature (7) of 5.8 per cent and 3.16 per cent for these two amino acids. Fromageot & Colas (8), using a newly developed method, reported that crystalline insulin contains 5.3 per cent aspartic acid and 19.3 per cent glutamic acid; these values are only in fair agreement with previous figures (7).

Sanger (9) has fractionated performic acid-oxidized insulin by several methods, precipitation at pH 6.0, salting out with 30 per cent ammonium acetate, or ionophoresis at pH 7.0. One fraction was acidic in properties and glycine was present as the terminal amino acid residues. Hydrolysis of this fraction and application of chromatography revealed the presence of leucine, isoleucine, valine, tyrosine, serine, glutamic acid, aspartic acid and cysteic acid. Absent from the hydrolysate, although present in insulin, were lysine, arginine, histidine, threonine and phenylalanine. The other fraction obtained from oxidized insulin had basic properties, and phenylalanine was present as the terminal amino acid residues. Amino acid analysis of this fraction

revealed all of the amino acids present in insulin. The data suggested that there are essentially two types of peptide chains in insulin, one basic and the other acidic, although the different chains of each type are probably not identical. These insulin oxidation products prepared by Sanger have been studied in the ultracentrifuge by Gutfreund & Ogston (10), who applied a new method for studying materials of low molecular weight. The acidic fraction had a diffusion constant of 17.6×10^{-7} and a molecular weight of 2,900; for the basic fraction, the values were 12.6×10^{-7} and 7,000, respectively. The former product appeared nearly homogeneous in the ultracentrifuge, whereas the latter was heterogeneous. The molecular weight calculated for the acidic fraction agreed well with Sanger's amino acid data (11), which gave an estimated molecular weight of 2,500. Assuming a weight of 12,000 for a four peptide chain subunit, the molecular weight (by difference) of the basic fraction should be about 3,100. The discrepancy between this value and that observed in the ultracentrifuge suggested that the basic fraction contained a considerable proportion of incompletely oxidized material, which would seriously affect the values of both the sedimentation and diffusion constants.

Sanger (12) has applied the performic acid oxidation method to insulin of several species: ox, pig, and sheep. There were no differences in the course of separation of the oxidation products into the two main fractions, one acidic and the other basic, thus indicating the same general overall structure. However, amino acid analyses of the acidic fractions revealed considerable differences in the contents of serine, glycine, threonine, and alanine. The most significant difference was the presence of threonine in the acidic fraction of pig insulin, and its absence from the same fraction of the oxidized ox and sheep insulins. These findings suggest that, contrary to general opinion, chemical differences do exist among highly purified insulin preparations of different species.

Butler and co-workers (13) have examined the possibility that the previously observed (14) slight hydrolysis of insulin by trypsin may have been due to the presence of approximately 0.5 per cent of chymotrypsin in the trypsin preparation employed. Incubation of recrystallized insulin with purified trypsin gave increases in nonprotein nitrogen which were the same as those seen in buffer alone, in the absence of enzyme. However, the insulin recovered from the incubation mixture showed a slight increase in free amino nitrogen and had an appreciably lower biological potency. It was suggested that trypsin breaks one or two bonds in the insulin molecule per unit of molecular weight 12,000, without liberation of measurable amounts of nonprotein peptide. The recovered insulin was also examined in the ultracentrifuge. Although the mean sedimentation constant appeared to be unchanged, there was a greater spread in the sedimentation rate curves, indicating an increased heterogeneity of the material, possibly due to a slight denaturation affecting the shape of the molecule and the way the submolecules aggregate. In the same laboratory, Phillips (15) attempted to apply partition chromatography to chymotrypsin digests of insulin. The protein hormone was digested under conditions which produced 50 to 65 per cent

of nitrogen soluble in 0.25 *N* trichloroacetic acid. After removal of the trichloroacetic acid and concentration of the soluble digest, the aqueous peptide-containing solution was subjected to filter paper partition chromatography. Using phenol and collidine as solvents, many of the peptides were spread out and could not be separated. However, normal butanol, saturated with five times diluted glacial acetic acid, produced several well-defined spots which gave characteristic ninhydrin colors. The data are generally difficult to interpret; certain of the fastest spots gave a green ninhydrin color which is similar to that seen when phenylalanine reacts with ninhydrin. Phillips points out that chymotrypsin requires a phenylalanyl or tyrosyl residue on the carboxyl side of a peptide bond in order that the latter be split. Reference is also made to unpublished data in which he has found that the undigestible, precipitated residue of insulin contains five aromatic amino acid residues, four tyrosine and one of phenylalanine, while the soluble peptide fraction, with a total combined weight of about 7,000, contains 10 residues, five of which are tyrosine and five phenylalanine.

Lens (16) has sought to elucidate the nature of the terminal carboxyl groups of insulin by digesting crystalline insulin with three times recrystallized carboxypeptidase at pH 7.7 in nonbuffered, aqueous medium. Digestion was continued until two to three amino groups had been liberated; this required a 4-hr. incubation period. Digestion was stopped by adjustment of the pH to 5, the solution filtered free from a precipitate which had formed, and ultrafiltered. In the ultrafiltrate, the total nitrogen was 3.80 moles per mole of insulin used; 1.24 moles were due to ammonia and the remaining 2.56 moles agreed well with the direct amino nitrogen determination of 2.6 moles in the ultrafiltrate. Hence, all of the amino groups liberated during proteolysis were accounted for in the ultrafiltrate which, besides some ammonia, did not contain any other form of nitrogen. Therefore, the enzyme actually liberated amino acids from the end of the peptide chain and no peptides had been formed. The ammonia liberated was not dependent on enzymatic action. The free amino acids in the ultrafiltrate were identified by paper partition chromatography. Only one amino acid, alanine, was detected. With a longer digestion period of 6 hr., glycine, valine, the leucine group, traces of tyrosine, and the mono-amino, dicarboxylic acid group were detected, in addition to alanine. It was concluded, therefore, that one and possibly three of the terminal amino acids with a free carboxyl group in the insulin subunit are alanine. After three alanyl groups have been liberated, at least six other amino acids appear almost simultaneously. The product obtained from the 4-hr. digestion period showed a total loss of biological activity and a complete failure to form the characteristic heat precipitate of insulin.

Inactivation of insulin also occurred when the hormone was incubated with arginase (17). The enzyme attacked the free guanidine groups of the protein, liberating urea. The pH optimum of the reaction was 9.0, and manganous ions increased the rate of the reaction. The rate of production of urea paralleled the degree of inactivation of the hormone.

Woolley (18) treated insulin with 2,4-dinitrofluorobenzene and digested the resulting derivative with pancreatin. On fractionation, the digests yielded four crystalline, yellow compounds. These same four crystalline compounds were also obtained from pancreatin digests of the dinitrophenol derivative of that fraction of performic acid-oxidized insulin which was soluble at pH 6. Evidence for the homogeneity of these products was presented. Amino acid analyses of each of these yellow derivatives revealed that they were of differing complexity. The data indicated that at least two distinct large peptides, with differing arrangements of amino acid residues, were produced by the pancreatic digestion of insulin.

Mirsky and co-workers (19) have described in some detail an insulin inactivating substance, termed "insulinase." The hormone was inactivated by incubation with the supernatant liquid from homogenized rat liver, kidney and muscle, and human, chicken, steer, and rabbit liver. On the other hand, the inactivating agent was not present in significant amounts in the supernatant liquid from homogenates of rat brain, whole blood, plasma, and red cells. The inactivating agent was destroyed at 80°; maximum inactivating action of insulin by the "insulinase" occurred at 37° and at pH 7.5. Precipitation of the inactivating substance occurred at half saturation concentration of ammonium sulfate. The activity was lost on dialysis but was restored on recombination of the two fractions; potassium, manganese, and magnesium ions restored the activity of the nondialyzable fraction. "Insulinase" was inactivated by cupric and zinc ions, by iodoacetate, and by iodobenzoate. Fasting produced a marked reduction of the insulinase activity of rat liver (20); the activity was restored to normal by feeding a balanced diet.

Stadie and co-workers (21) developed an experimental technique whose application gave evidence of a direct chemical combination of insulin with the intact muscle cells of the rat diaphragm. This combination was seen with a variety of insulin preparations. The effect was demonstrated by equilibrating the diaphragm of a normal rat for 1 min. in a phosphate-saline medium containing 0.1 unit of insulin per ml. of medium. The diaphragm was then removed and washed two times in a large volume of medium. The equilibration of this diaphragm in a medium containing glucose, but no added insulin, resulted in as much glycogen production in the diaphragm as in control diaphragms to which insulin was added. On the other hand, control diaphragms not exposed to insulin synthesized approximately 50 per cent less glycogen when compared with diaphragm muscle previously exposed for 1 min. to insulin. The extent of the combination of insulin with the diaphragm muscle did not vary significantly over the pH range of 5.7 to 8.4. The effect was greater at 38°C. than at lower temperatures. The combination also occurred under anaerobic conditions. It was suggested that a chemical reaction takes place between insulin and some muscle constituent. This suggestion was further supported by observations that the effects of insulin are dependent upon the time of contact of the diaphragm with insulin, the concentration of insulin, and on the temperature during equilibration. In

a second paper, the same investigators (22) studied various hormonal influences on the described chemical combination of insulin with rat diaphragm muscle. The metabolic state induced by injection of alloxan in the rat diminished the ability of diaphragm muscle to combine with insulin. On the other hand, adrenalectomy and hypophysectomy had no effect on the combination. Intraperitoneal injection of 3 ml. of crude anterior pituitary extract in a rat 20 hr. prior to the removal of the diaphragm completely impaired the ability of the diaphragm to combine with insulin. However, the injection of adrenal cortical extract had no such effect. Highly purified and crystalline growth hormone, when injected prior to removal of the diaphragm, was effective in small doses in preventing this combination. The insulin-diaphragm combination effect was completely blocked by 0.5 mg. of crystalline growth hormone, injected 20 hr. previously. The effect of crude anterior pituitary extracts was also demonstrable *in vitro*. On the other hand, there was no *in vitro* effect of purified growth hormone preparations.

Lowell & Franklin (23) reported evidence suggesting that treatment of insulin with cysteine in a manner which abolished hypoglycemic activity did not alter the capacity of the protein to combine with antibody elicited by injection of the untreated, biologically active protein.

Pituitary Hormones

Lactogenic hormone.—Using microbiological and chemical methods, Li (24) achieved a complete amino acid analysis of highly purified lactogenic hormone isolated from whole sheep pituitaries. The values obtained in gm. per 100 gm. of protein were: arginine, 8.6; aspartic acid, 11.6; glycine, 4.0; histidine, 4.5; lysine, 5.3; phenylalanine, 4.1; proline, 6.2; serine, 6.5; tyrosine, 4.7; glutamic acid, 14.1; isoleucine, 7.2; leucine, 12.5; methionine, 3.6; valine, 5.9; threonine, 4.8; tryptophane, 1.2; cystine, 3.1; and amide nitrogen, 1.0. The 17 amino acids and the amide nitrogen have accounted for 99.9 per cent of the protein nitrogen. The minimum molecular weight of the lactogenic hormone, calculated from the concentration of five amino acids, averaged $33{,}287 \pm 226$. This value is close to the previous figure obtained by ultracentrifugation (25), but is somewhat higher than that calculated from osmotic pressure (26) and diffusion (27) measurements.

Growth hormone.—Smith *et al.* (28) have published an ultracentrifugal study of the crystalline growth hormone obtained from bovine anterior pituitary glands (29). At pH 7.0, the protein tended to produce aggregates of larger size. At pH 9.5, the data indicated a single molecular species. From the sedimentation and diffusion constants, 3.6 S. and 7.36×10^{-7}, respectively, a molecular weight of 49,200 was calculated. A study by Li & Moskowitz (30) of the behavior of purified growth hormone (31) in the ultracentrifuge at pH 9.1 or pH 9.7 indicated that the protein was essentially monodisperse. A sedimentation constant of 3.1 S. gave a calculated molecular weight of 44,000.

Adrenocorticotropic hormone.—Chase (32) presented immunological and physiological evidence for the development of antibodies, with antihormonal activity, in the blood of mice as a result of the chronic injection of highly purified adrenocorticotropic hormone (ACTH).

Li (33) published an interesting preliminary report regarding the suggestion that ACTH, isolated from sheep pituitary glands, preserves its hormonal activity after partial acid or peptic hydrolysis. The active peptide fragments were not precipitable by trichloroacetic acid and were readily dialyzable. The average peptide length in the active hydrolysates was reported to be approximately 8. When the hormone was digested at 37.4° with crude pepsin to the extent of 48 to 51 per cent, and approximately 10 per cent of the total nitrogen in the hydrolysate was present as amino nitrogen, the hydrolysate was still adrenocorticotropically active. No free amino acid could be detected in the hydrolysate by the ninhydrin reaction. No yields of activity are given in comparison with the original starting product. In view of the great physiological and therapeutic interest in ACTH, further details of the chemical nature of the active peptide(s) are of prime importance.

It is of some historical interest that following the discovery of epinephrine and the isolation of thyroxine from thyroidal protein, all efforts to obtain biologically active moieties from protein hormones have, to the present time, been unsuccessful. Years of experimental work have been required to permit acceptance of the conclusion that the biocatalytic action of insulin is dependent on the intactness of the protein structure. Once this gained recognition generally, it appeared logical to apply similar reasoning to other protein hormones, notably those of the anterior pituitary gland. It would now appear that the rigidity of thinking in this field must be altered, at least in the case of ACTH, and due consideration given again to the possible existence in these proteins of active centers or groupings, some of which can be isolated.

Gonadotropic hormones.—Employing purification procedures which involved a combination of ammonium sulfate and cold ethanol fractionation, Li, Simpson & Evans (34) accomplished the isolation from sheep pituitaries of a protein which caused follicle development only in the ovaries of hypophysectomized rats and which behaved as a single substance when studied in the electrophoresis apparatus and in the ultracentrifuge. The purified protein showed no evidence of other types of biological activity.

Whitten (35) reported that pituitary and chorionic gonadotropins were rapidly inactivated by Lee-B influenza virus and by receptor-destroying enzyme preparations (*Vibrio cholerae*). The data suggested that the follicle-stimulating component of the crude pituitary gonadotropic preparation was destroyed. Enzyme preparations from *Clostridium welchii* rapidly inactivated serum gonadotropin (36).

Oxytocin.—By application of the countercurrent distribution principle of Craig (37), Livermore & du Vigneaud (38) have purified the oxytocic fraction of the posterior pituitary gland. A product having a potency of

865 units of oxytocic activity per mg. was obtained from a starting material of approximately 20 units per mg. The characteristics of the distribution curve of this high potency product suggested either that it was very nearly pure or that if any impurity were present, it had a distribution coefficient almost identical with that of the hormone. The ultraviolet absorption spectrum of a solution of purified oxytocic material in 0.05 per cent acetic acid showed strong end absorption below 250 mμ and a small peak at 275 mμ that coincided with the absorption peak of tyrosine.

Wachtel (39) described the preparation of a lipid-soluble fraction from the acetone extract of posterior lobes of cattle pituitary glands. On subcutaneous injection of this fraction in dogs, an increase in the blood cholesterol was observed. Two other fractions were described, one lowering the serum phospholipids and the other increasing the total serum lipids. The cholesterol-depressing fraction was separated chromatographically into three fractions, two of which were claimed to lower specifically the blood levels of esterified and free cholesterol, respectively.

Hyperglycemic-Glycogenolytic Factor

The single important contribution to the chemical nature of the hyperglycemic-glycogenolytic factor which has appeared since this subject was reviewed last year is that by Sutherland and co-workers (40). These investigators have purified the hyperglycemic-glycogenolytic factor from both pancreas and dog gastric mucosa. The pancreatic product employed as starting material was either highly purified amorphous insulin or crystalline zinc insulin. The hyperglycemic-glycogenolytic factor could be purified by forming insulin fibrils according to the method of Waugh (41). These fibrils were removed by filtration; the filtrate contained an increased amount of the hyperglycemic-glycogenolytic factor per mg. of protein. Fractionation of alkali-inactivated insulin also yielded a 10-fold increase in hyperglycemic-glycogenolytic activity. Fractionation of extracts of the upper two-thirds of the gastric mucosa of the dog was achieved by the same methods of extraction, purification, and precipitation that were successful in the purification of the hyperglycemic-glycogenolytic factor of pancreatic origin. The evidence indicated that the two factors (hyperglycemic and glycogenolytic) are closely related, if not identical. The protein nature of the active fractions was strongly suggested in that the classical methods of protein fractionation could be employed in the concentration of the hyperglycemic-glycogenolytic activity, and the latter activity was not lost by dialysis. Electrophoretic studies revealed that a component comprising approximately 10 per cent of the total protein, and migrating slightly slower than insulin, could be detected in the electrophoretic patterns of amorphous and of crystalline zinc insulin in acetate buffers at pH 3.8. The identity of this slower component with the hyperglycemic-glycogenolytic factor was suggested in that this component was not present in electrophoretic studies of insulin preparations which are free of the factor.

Epinephrine-Norepinephrine

Further evidence has been obtained that the pressor activity in extracts from adrenal glands is not due to the presence of epinephrine alone but also to the presence of norepinephrine. This had been demonstrated for the pig's gland by Schümann (42) and for the dog's adrenal gland by Bülbring & Burn (43), using biological methods. The presence of both of these pressor amines in extracts of adrenal glands from cattle has now been demonstrated by von Euler & Hamberg (44), who compared the results obtained by biological methods with those obtained by quantitative paper chromatography. The same laboratory reported (45) the isolation of a mixture of L-norepinephrine and L-epinephrine, in approximately 1 to 4 proportions, from the crude protein-free extract of cattle adrenals with the aid of ion exchange resins. The bases were then separated by counter-current distribution between 0.02 *N* hydrochloric acid and phenol. Extraction of the phenol with ether and addition of ammonia yielded pure crystalline L-norepinephrine. An additional valuable contribution in this field by von Euler & Hamberg (46) has been the development of a colorimetric method for the estimation of norepinephrine in the presence of epinephrine. The method is based upon the differences in rates of oxidation of the two amines with iodine, depending on the hydrogen ion concentration of the solution. The chemical method gave results which agreed well with those obtained by biological assays.

Goldenberg and co-workers (47) have also demonstrated the presence of norepinephrine in commercial extracts of adrenal glands obtained from cattle; both chemical and biological methods were employed. These investigators concluded that the samples assayed showed 12 to 18 per cent of norepinephrine; one sample contained as much as 36 per cent of this substance. Tullar (48) has isolated L-norepinephrine from such extracts. Large amounts of norepinephrine have been found by Holton (49, 50) in tumors of the adrenal medulla; 1 gm. of tissue contained approximately 3.5 mg. of epinephrine and 6.0 mg. of norepinephrine. The presence of both amines in these tumors was confirmed by their color reaction with polyphenolase and by paper chromatography. Beauvallet & Brochart (51) have reported that tungstic acid filtrates of seminal plasma and of washed spermatozoa both contained a small amount of norepinephrine.

Bülbring (52) demonstrated the *in vitro* methylation of norepinephrine by suspensions of ground dogs' or cats' adrenals during a 1-hr. incubation period at 37°C. The presence of adenosinetriphosphate was essential for this methylation. Adrenal glands removed after prolonged splanchnic stimulation appeared to have a higher methylating capacity than glands taken from a freshly killed animal. Bülbring & Burn (53) established the formation of epinephrine from norepinephrine during perfusion of the adrenal gland of the dog with heparinized blood to which norepinephrine had been added. During the perfusion, there was an increase in the amount of epinephrine coinciding with a decrease in the amount of norepinephrine. These data are in ac-

cord with the previously mentioned *in vitro* methylation studies. Holtz & Kroneberg (54) reported that slices of hog, beef, and guinea pig adrenals, incubated in phosphate buffer under aerobic conditions, converted hydroxytyramine and tyramine into a substance with the physiological properties of epinephrine. Since phenylhydroxyethylamine was not transformed to epinephrine under the same experimental conditions, the authors suggested that oxidation of the ring must precede transformations of the sidechain.

Thyroid Hormones

Roche and co-workers (55) have studied the purification of thyroglobulin. Different samples of thyroglobulin, carefully purified by the usual precipitation methods, were found to differ considerably in iodine content. Solubility studies (56) in neutral salt solutions of the proteins of crude thyroid extracts of the ox, horse, dog, and pig led to the identification of three fractions of thyroglobulin, which could be separated on the basis of their solubility properties. The latter properties appeared to be independent of the content of iodine or of iodinated amino acids. Highly purified pig thyroglobulin examined in the ultracentrifuge showed (57) a sedimentation constant of 19.4 S.; this value, together with a diffusion constant of 2.60×10^{-7} and a partial specific volume of 0.723, gave a calculated molecular weight of 650,000. Despite the fact that the protein appeared to be homogeneous as judged by ultracentrifugation and electrophoresis studies, it was demonstrated to contain three components when studied in solvents of high ionic strength. The following amino acid concentration per 100 gm. of protein is reported for this preparation: arginine, 12.7; histidine, 2.2; lysine, 3.4; phenylalanine, 6.7; tryptophane, 2.1; tyrosine, 3.1; diiodotyrosine, 0.5; thyroxine, 0.21; cystine, 3.6; methionine, 1.3; alanine, 7.4; glycine, 3.7; leucine, 12.8; valine, 1.45; and serine, 10.8. Thyroglobulins of normal pigs, cattle, and dogs showed surprisingly small differences in amino acid composition. On the other hand, the thyroid protein of each species was observed to contain varying amounts of iodine despite the constancy of the content of tyrosine and other amino acids. The suggestion was made that the formation of the thyroid protein, and its iodination, are two independent processes. In thyroglobulins of goitrous animals (swine and beef with hypertrophied thyroids), elevated tyrosine values and lower cystine values were seen.

Hird & Trikojus (58) used paper chromatography to examine the acid insoluble fraction from hydrolysates of artificially iodinated proteins for the presence of iodine-containing substances. In two samples of different origin, thyroxine and diiodothyronine were found, as well as a third substance thought to be 3,3′,5-triiodothyronine. Taurog, Chaikoff & Tong (59) confirmed the previous observation by Fink & Fink (60) indicating the presence of monoiodotyrosine in the thyroid gland. Studying the problem with the aid of radioactive iodine, and using two dimensional filter paper chromatograms, Taurog and co-workers demonstrated that diiodotyrosine and monoiodotyrosine were present in the alkaline hydrolysates of the thyroids of rats injected with radioactive iodine and the glands removed 10 min., 24 hr. and

48 hr. after iodine injection. The rate data obtained indicated that monoiodotyrosine is a precursor of diiodotyrosine. These same investigators (61) also reported that about 15 per cent of injected radioiodine was located in the thyroxine of the thyroid glands of rats injected 24 hr. previously with radioactive iodine. A significant amount of the isotope was found in the diiodotyrosine and inorganic fractions. The analytical data obtained from the inorganic fraction indicated an appreciable breakdown of organic iodine during hydrolysis of the glands.

Tishkoff and co-workers (62) also studied chromatograms of hydrolysates of the thyroids of rats injected with radioiodine. Thyroxine, monoiodotyrosine and 3,5-diiodotyrosine were present, with diiodothyronine indicated as present. Three spots on the filter paper chromatogram were unidentified iodine compounds. One of these reacted with ninhydrin. The presence of a characteristic iodide double spot suggested that free iodide was formed during alkaline hydrolysis, or that the thyroid gland contains undialyzable iodide in bound form. Simple extraction of thyroid glands with collidine-lutidine revealed in the extracts the presence of radioactive material corresponding to free iodide, and to mono- and diiodotyrosine. No thyroxine was detected. It was suggested that mono- and diiodotyrosine exist partially in free form in the gland and may not be bound completely to protein.

Pitt-Rivers and Roche and co-workers have continued their studies of the factors influencing thyroxine formation during the iodination of proteins *in vitro* and their subsequent hydrolysis. Roche & Michel (63) confirmed their previous observation (64) that blocking of the carboxyl and the amino group of tyrosine did not influence thyroxine formation. The length of the amino acid chain in the substituted tyrosine group was of considerable significance since 26 per cent of an L-leucyl-L-tyrosine preparation was converted to new combined thyroxine under the experimental conditions employed. However, only traces of thyroxine were formed from glycyl-L-tyrosine. Pitt-Rivers (65) also found that blocking of the amino group by acetylation, and the carboxyl group by a peptide linkage, favored a greater conversion of diiodotyrosine derivatives to thyroxine analogues at the pH of body tissues. Thus, N-acetyl-L-diiodotyrosine gave 15 to 20 per cent of N-acetyl-L-thyroxine. N-acetyl-DL-diiodotyrosylglutamic acid gave 36 per cent N-acetyl-DL-thyroxylglutamic acid.

Roche and co-workers (66) concluded that only a portion of the tyrosine in casein, insulin, thyroglobulin, and zein could be transformed into thyroxine. The major portion of the tyrosine was obtained as diiodotyrosine. Since the maximum yield of thyroxine from each protein did not always bear the same relation to the tyrosine content of the protein, it appeared that the structure of the protein, and the positions of the tyrosine residues in this protein, affect the yield of thyroxine. This suggestion is in agreement with the observation of Michel & Pitt-Rivers (67) that, despite the high tyrosine content of silk fibroin, iodination of the protein (in cupriethylene diamine solution), followed by hydrolysis, yielded only minute amounts of thyroxine. Since the poor yield could not be explained on the basis of insolubility of the

silk fibroin, it was suggested that the close proximity of the diiodotyrosine residues to one another, in the iodinated protein, resulted in undesirable steric effects.

Roche and co-workers (68) found that iodocaseins prepared by various methods and containing nearly the same contents of thyroxine were of very different activities when given by mouth, indicating a difference in degree of digestion and assimilation. Also, iodoinsulin, like natural thyroglobulin, showed per unit weight of thyroxine a greater biological activity than free thyroxine. The data of Frieden, Tuckich & Winzler (69) also re-emphasize the marked effect of digestion and absorption on the biological activity of thyroxine and of thyroid preparations. DL-Thyroxine exhibited half its parenteral activity when administered orally as the soluble sodium salt. Four different commercial thyroid preparations showed 70, 71, 72 and 98 per cent of their parenteral potency, and the latter was greater than could be accounted for on the basis of the L-thyroxine content of the test substances. Pitt-Rivers (70) found that the amount of thyroxine isolated from iodinated proteins was proportional to the biological activity of the proteins. Griesbach and co-workers (71) reported that D-thyroxine has 0.3 the biological activity of the L-thyroxine in the rat.

Both Pitt-Rivers (72) and Formijne (73) have demonstrated that antithyroid substances, e.g., thiourea, inhibit the *in vitro* iodination of proteins. In addition, Pitt-Rivers (72) observed that a variety of goitrogenic substances inhibited the formation of acetylthyroxine from acetyldiiodotyrosine.

Several groups of investigators have studied the thyroid-like and antithyroid-like activity of a number of compounds. Kennedy & Griesbach (74) found that 3,3′,5,5′-tetrabromothyronine, the bromine analogue of thyroxine, was effective in preventing the development of thyroid and pituitary changes in rats receiving methyl thiouracil. The bromine derivative had about 0.05 the biological activity of DL-thyroxine. Frieden & Winzler (75) have described a number of derivatives of 3,5-diiodotyrosine and 3,4-diiodo-4-hydroxybenzoic acid. These compounds have been tested for thyroxine antagonism, as indicated by the effects upon tadpole metamorphosis. The benzylethers of the substances reduced the effect of thyroxine by 50 per cent, when the molar ratio of inhibitor to thyroxine was 8 to 37. These ethers were also competitive inhibitors of the thyroxine-like active compound, 3,5-diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid, in ratios similar to those observed with thyroxine. However, even at very high molar ratios, these benzylethers had no effect on the thyroxine-like activity of 3,5-diiodo-4-(4′-hydroxyphenoxy)-aniline. Therefore, these compounds with thyroxine-like activity are probably not converted to thyroxine for activity. It would appear that considerable modification of the structure of thyroxine can result in the production of potent competitive antagonists, which appear to have the same lack of specificity with regard to the side chain that has been found (76) for thyroxine-like activity. It is possible that the thyroxine or thyroxine-like active molecule associates with an enzyme through its side chain and exerts its function by means of the orthodiiodohydroxyphenyl

ether structure at the opposite end of the molecule. Maclacgan and coworkers (77) confirmed the above-reported thyroxine-inhibiting activity of the benzyl ether of 3,4-diiodo-4-hydroxybenzoic acid by studying its effect on oxygen consumption in mice. A much higher ratio of inhibitor to thyroxine was required than was found when the tadpole was the test animal. These authors described a slightly more effective inhibitor, 3,5-diiodoanisaldehyde dimethylacetal.

Cortell (78) tested seven thyroxine analogues in rats treated with thiouracil. Of considerable interest was the observation that 3′-fluoro-5′-iodo-3,5-diiodothyronine had a thyroxine-like activity close to that of thyroxine. Thus, the presence of only one iodine atom in the second ring is essential for biological activity. One compound, 2′,6′-diiodothyronine, was able to antagonize thyroxine action in a dose 150 times that of the thyroxine and also showed inhibitory action against injected thyroglobulin. Further evidence was obtained of the greater activity of thyroglobulin than thyroxine, as calculated on the basis of the protein's content of thyroxine iodine.

One of the most active of the recently tested antithyroid compounds has been isolated from natural sources. Astwoood, Greer & Ettlinger (79) have isolated from the root and seed of turnip and from the seed of cabbage, kale, and rape a crystalline substance with marked thyroid inhibiting activity. When tested in normal human subjects by use of radioactive iodine, the compound was found to have an antithyroid activity equal to that of thiouracil. The structure of the compound was established as *l*-5-vinyl-2-thiooxazolidone.

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THE WATER-SOLUBLE VITAMINS

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No slackening in the interest in vitamins is yet apparent, and new factors of dietary importance for animals or microorganisms continue to be discovered at an apparently increasing rate. The present review covers only a portion of the literature on these subjects which has appeared during the past year. Because of space limitations and the lack of fundamental advances, no consideration of ascorbic acid is given.

Thiamine

Horwitt & Kreisler (130), in a study of human patients receiving controlled amounts of thiamine over prolonged periods, found that consistent rises in blood levels of lactic and pyruvic acids occurred only after other clinical symptoms of thiamine deficiency were present, and were useless as an index of mild thiamine deficiency. Such mild deficiencies could be detected by use of an "index of carbohydrate metabolism" (CMI) which related the amount of glucose, lactic acid, and pyruvic acid found in the blood following glucose administration and a mild exercise test (stair climbing) according to the formula: $\text{CMI} = (L-(G/10)+15P-(G/10)) \div 2$, where L, P and G are the milligrams of lactic acid, pyruvic acid, and glucose respectively found in 100 cc. of blood. Indices below 15 were found in all persons with sufficient thiamine intake: in mild thiamine deficiency indices somewhat higher than 15 were consistently found. Kirk & Chieffi (160) determined the fasting levels of thiamine and pyruvic acid in the blood of 220 individuals between 40 and 102 years of age. Values for thiamine showed great variation, but there was a slight tendency to a lowering with advancing age. Blood pyruvate levels, in contrast, did not vary with age.

Holt *et al.* (129) found that thiamine excretion in human infants approached a relatively constant minimum level at lower levels of thiamine intake. The amount of thiamine necessary to cause excretion just above this minimum value varied between 0.14 and 0.20 mg. per day in four infants, and is considered to represent the thiamine requirement.

A reinvestigation by Scott & Verney (252) of the appetite for thiamine in rats revealed that when deficient, most of these animals selected thiamine-containing diets in preference to thiamine-free diets. Such preference was not displayed by normal animals, and was not established until the animals were distinctly deficient as judged by reduced weight gains and food intake.

Oxythiamine reacts like thiamine toward the Prebluda-McCollum re-

agent [Soodak & Cerecedo (278)], but does not give the thiochrome reaction. Like thiamine, it is destroyed by sulfite, but not by sodium in glacial acetic acid; and is adsorbed and eluted from Decalso. Frohman & Day (82) found that 4 hr. after injection of 150 mg. of oxythiamine into adult rats there was a marked rise in blood levels of pyruvic and lactic acids, with the shift toward pyruvate characteristic of severe thiamine deficiency. Seventy-five milligrams of thiamine was insufficient to overcome the effects of 150 mg. of oxythiamine; equal or greater amounts of thiamine, however, completely prevented the action of this antimetabolite. The excretion of thiamine by rats ingesting 24 mg. of this vitamin daily was raised by 100 to 175 per cent within one or two days following injection of 50 mg. of oxythiamine. The extra thiamine excreted equalled or surpassed the oxythiamine injected. Thus, there was a true displacement of thiamine from the tissues by oxythiamine, and since 50 mg. of oxythiamine will displace 50 mg. of thiamine, this product appears to be one of the most potent antithiamines known.

The product first synthesized by Tracy & Elderfield (306), and named "pyrithiamine" by Woolley & White (350), was found by Wilson & Harris (341) to be a mixture of compounds of undefined structures which contained the pyrimidine and pyridine moieties in ratios of 1:2, 1:3, 1:4, etc., and which did not have the properties (absorption spectrum, etc.) to be expected of this thiamine analogue. A modified synthesis resulted in the true analogue of thiamine containing the 2-methyl-3-β-hydroxyethylpyridine residue in place of the thiazole moiety of thiamine. This has been named "neopyrithiamine," to distinguish it from the earlier product, and was found by Emerson and associates [quoted in (341)] to be approximately four times as effective as pyrithiamine in antagonizing the action of thiamine in rats.

Thiamine destruction by the Chastek paralysis enzyme (thiaminase) is inhibited by *o*-aminobenzyl-4-methylthiazolium chloride in a fully competitive manner. *o*-Aminobenzyl-2-methylthiazolium chloride, and *o*-aminobenzyl-2,4-dimethylthiazolium chloride also inhibit the reaction. *p*-Aminobenzylthiazolium compounds were inert toward the system, while *m*-aminobenzylthiazolium compounds caused increased destruction of thiamine [Sealock & Livermore (255)]. *m*-Nitroaniline and *m*-aminobenzoic acid also activate the enzyme, and analysis of the mechanism of their action showed that during thiamine cleavage, the pyrimidine moiety of thiamine was not liberated as the alcohol, but was transferred to the amino group of the activator [Sealock & Davis (254)]. When *m*-nitroaniline was the activating amine, its condensation product with the pyrimidine moiety of thiamine was isolated in analytically pure form from the reaction mixture. The authors view the activating amines as substitutes for an unidentified, naturally occurring amine to which the pyrimidine may normally be transferred, and point out that the reaction may represent an enzymatic transmethylation with a substituted methyl group. Neopyrithiamine also is split by thiaminase but at a much slower rate than is thiamine [Sealock & White (256)].

Thiamine triphosphoric acid (TTP, analogous in structure to adenosinetriphosphate) activated the apocarboxylase of baker's yeast, but was only

$\frac{1}{4}$ to $\frac{1}{3}$ as active as cocarboxylase at low concentrations. At saturation, the resultant decarboxylase system was about 80 per cent as active as that found with excess cocarboxylase. TTP was hydrolyzed by potato apyrase to yield cocarboxylase and finally thiamine monophosphate, but was not hydrolyzed by the adenosine triphosphatase of muscle [Velluz *et al.* (316)].

The distribution of thiamine in brain tissue (319), and in the embryo of incubating hen eggs (253) has been described.

Riboflavin

Determination (12), distribution (33, 34, 44, 233), and retention of riboflavin in the cooking of foods (200, 201) have received attention during the past year.

Griffin & Baumann (100) studied the incidence of tumor formation induced in rats by the use of the azo dyes, *p*-dimethylaminoazobenzene and *m'*-methyl-*p*-dimethylaminoazobenzene, when the diets fed varied in their content of riboflavin. As anticipated, the hepatic stores of riboflavin paralleled the riboflavin content of the diets fed. The carcinogens lowered the level of riboflavin in the liver and the effectiveness of the azo dyes as carcinogens paralleled their effectiveness in lowering hepatic riboflavin. Thus an inverse relationship was present between the rate of tumor development and the level of hepatic riboflavin. Similar results were obtained by Kensler (154) who noted further that the administration of biotin or adenine with *p*-dimethylaminoazobenzene did not alter its effect on hepatic stores of riboflavin or destruction of the carcinogen by liver slices.

Griffin *et al.* (101) found that diets containing 24 per cent of casein or 12 per cent casein plus 0.4 per cent methionine afforded greater protection against carcinogenic dyes and better retention of riboflavin in the liver than did unsupplemented diets containing 12 per cent casein.

Stoerk & Emerson (287) have observed complete regression of established transplants of lymphosarcoma (6C3H-CD) in mice during riboflavin deficiency or during the administration of the riboflavin antagonists, isoriboflavin or galactoflavin. Fifteen of 48 mice made temporarily riboflavin-deficient by a diet low in riboflavin or by administration of an antagonist, showed complete regression of transplants and survival for 200 days without recurrence of the tumors, despite the administration of sufficient amounts of riboflavin for maintenance. Mice that had survived for 60 days following the original tumor transplant when reinoculated with the lymphosarcoma tissue failed to "take" the second implant.

In studies on the effect of visible light on etiolated peas, Galston & Hand (86) observed that addition of riboflavin to the medium resulted in a growth inhibition that could be reversed partially with indoleacetic acid. *In vitro* experiments (84) demonstrated that riboflavin sensitized the photooxidation of various indoles including tryptophane, indolebutyric, and indoleacetic acids. The latter results may indicate the mechanism of the inhibitory action of riboflavin *in vivo*.

Galston & Baker (85) found that various enzyme preparations similarly

were inactivated by visible light in the presence of riboflavin. Presumably this inactivation results from riboflavin-sensitized photooxidation of tryptophane in the enzyme molecule.

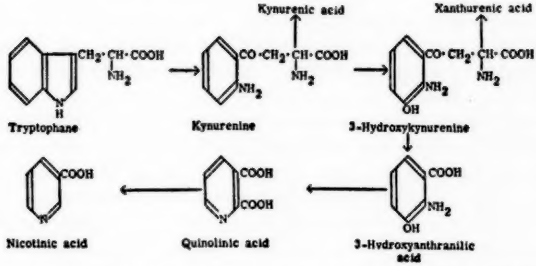
Garber *et al.* (87) showed with human subjects that the absorption of riboflavin, in contrast to that of thiamine, was not decreased by the ingestion of live yeast. Mayfield & Hedrich (183) place the daily riboflavin requirement of the rat for maximum efficiency of protein utilization at about 5 μg.

Bessey, Lowry & Love (12) have applied their fluorometric method to a study of the distribution of riboflavin, riboflavin monophosphate, and riboflavin dinucleotide in tissues. The latter two compounds account for practically all of the riboflavin present. Riboflavin adenine dinucleotide accounted for 70 to 90 per cent of the total riboflavin. These data are not wholly in agreement with an earlier report of Crammer (49) who could demonstrate no free riboflavin or riboflavin mononucleotide in several rat tissues by paper chromatography.

Hou reported (131) conversion of riboflavin to lumichrome during the growth of the tubercle bacillus.

Nicotinic Acid

Ingestion of tryptophane by all higher animals so far investigated leads to enhanced excretion of nicotinic acid and its metabolites, and interest in the mechanism and nutritional significance of this conversion remains high. Haskins & Mitchell (110) demonstrated that 3-hydroxykynurenine can be utilized in place of nicotinic acid by certain mutant strains of *Neurospora;* in the series of chemical transformations by which *Neurospora* transforms tryptophane to nicotinic acid, this substance fits logically between kynurenine and 3-hydroxyanthranilic acid. The nature of another intermediate in the conversion has been clarified through the isolation of quinolinic acid from the urine of tryptophane-fed rats by Henderson (117). Singal *et al.* (265) had first noted a substance in the urine of rats fed tryptophane which showed nicotinic acid activity for *Lactobacillus arabinosus* after, but not before, treatment with acid. Since this substance did not appear in the urine of animals fed nicotinic acid, it was postulated that the substance might be a precursor of nicotinic acid. Henderson (117) showed that this substance was quinolinic acid, which is readily decarboxylated to nicotinic acid by treatment with acid. In the conversion of tryptophane to nicotinic acid, quinolinic acid would appear to be a logical immediate precursor of nicotinic acid, and would result through oxidative cleavage of the benzene nucleus of 3-hydroxyanthranilic acid between carbon atoms 3 and 4, followed by closure of a pyridine ring between carbon atom four and the amino group. The probable course of the reaction may be visualized from the figure shown on p. 281. Whether quinolinic acid is an actual intermediate in the conversion, or whether it is a stabilized excretory product of such an intermediate, is not known. Henderson (117) cites unpublished data, however, to show that quinolinic acid does relieve nicotinic acid deficiency in the rat. Inclusion of 3-hydroxykynurenine in the above scheme provides a mechanism for forma-



tion of xanthurenic acid fully analogous to that by which kynurenic acid is presumably formed.

Heidelberger *et al.* (115, 116) have shown that the above scheme is fully compatible with the disposition by mammals of tracer atoms incorporated at various positions into the tryptophane molecule. That nicotinic acid arises from tryptophane in mammals by a mechanism closely analogous to that first established in *Neurospora* thus appears proved. A previously discussed report (314) indicated that kynurenine was inactive in rats as a precursor of nicotinic acid. A subsequent investigation [Kallio & Berg (147)] shows, however, that kynurenine is active in the rat, as it is in *Neurospora.*

Present evidence indicates conclusively that conversion of tryptophane to nicotinic acid is not dependent upon intestinal microorganisms, but is carried out in the tissues of mammals. Henderson & Hankes (118) and Hundley (135) removed the entire intestinal tract of rats and found that such animals still excrete additional N′-methylnicotinamide in response to injections of tryptophane. Tryptophane administered to infants by intravenous drip over several hours resulted in excretion of amounts of N′-methylnicotinamide as large as those produced by oral administration of the same dose of tryptophane [Snyderman, *et al.* (277)]. Previous results showing a lowered efficiency of conversion following injection of tryptophane, as compared to oral administration, were explained on the basis of intestinal synthesis following oral administration. They are believed by these authors to be due to a flooding of the conversion mechanisms by excess tryptophane administered by injection, with consequent disposal by mechanisms which do not operate to the same extent when tryptophane is supplied more slowly. Schweigert (250) found that incubation of 3-hydroxyanthranilic acid with rat liver slices gave rise to increased quantities of nicotinic acid as indicated by assay with *L. arabinosus* following acid hydrolysis. Tryptophane was not converted to nicotinic acid under the same conditions. By a slightly different technique, including more vigorous aeration and the presence of glucose, Hurt *et al.* (136) found increased nicotinic acid after incubation of liver slices with tryptophane. The hydrolytic procedures employed by both groups would not differentiate between nicotinic acid and quinolinic acid; Schweigert & Marquette (251) subsequently showed that the product formed from 3-hydroxyanthranilic acid was chiefly quinolinic acid.

To conclude that mammalian tissues convert tryptophane to nicotinic acid is not, of course, to deny that intestinal flora may also do so and contribute thereby to the supplies of this vitamin available to the host animal. Ellinger & Abdel Kader (61, 62, 64) have shown that cultures of *E. coli* synthesize nicotinamide, as do the mixed cultures from the caecum of the rat. Such synthesis was increased 300 to 400 per cent by the addition of ornithine to the culture medium, and to a lesser extent by additions of arginine and glutamine; under the conditions used, these supplements had little or no effect on growth or acid production. Supplementation with tryptophane did not enhance nicotinamide synthesis in *E. coli*, but did enhance synthesis by the mixed cultures, although to a lesser extent than ornithine. Ornithine and tryptophane together were no more effective than ornithine alone. These and other results led these authors to believe that ornithine serves the bacteria as a precursor of nicotinamide, and that tryptophane functions only indirectly by stimulating ornithine formation (64). Such a hypothesis was, however, proved untenable by the finding that various methyltryptophanes reduced nicotinamide synthesis by these bacteria to zero even though ornithine was present (62). The latter finding emphasizes the probable role of tryptophane as the precursor in these bacteria and leaves unexplained the peculiar effectiveness of ornithine in enhancing the synthesis. The latter compound was long ago found to increase synthesis of trigonelline by certain higher plants, and was postulated as an intermediate in the synthesis on these grounds. Nason (195), however, has provided evidence that tryptophane also serves as the precursor of nicotinic acid in the growing embryos of the corn plant.

Species differences in the metabolism of nicotinic acid are known to exist. Ellinger & Abdel Kader (60) employed a differential method for the determination of nicotinic acid, nicotinamide, nicotinuric acid, N′-methylnicotinamide, and trigonelline to measure the excretion of these substances in the urine of man, dog, cat, rat, guinea pig, and rabbit following ingestion of either nicotinic acid, nicotinamide, or coramine. These animals could be divided into two groups: man, cats, dogs, and rats excreted N′-methylnicotinamide as the principal product regardless of the form of the vitamin fed; nicotinamide, but only small amounts of nicotinic acid, was also excreted in varying proportions. Rabbits and guinea pigs, by contrast, excreted principally nicotinic acid, regardless of the form of the vitamin fed, and essentially no N′-methylnicotinamide. Surprisingly, nicotinuric acid was not encountered in any of these urines; this compound was rapidly split by the dog and rat. An unidentified metabolite of nicotinic acid, which, like N′-methylnicotinamide gives a fluorescent product with acetone, but which failed to give the König reaction was detected in urine of guinea pigs and rabbits. Recoveries of excreted metabolites were lower in man than in any other species, presumably due to formation of the 6-pyridone of N′-methylnicotinamide, which was not determined.

The characteristic excretion of nicotinic acid by the rabbit was not altered by feeding a meat diet (63), a fact which emphasizes that this is a spe-

cies difference, and not one due to dietary differences. Additional herbivora (sheep, goats, and calves) were found by Pearson *et al.* (214) to excrete much more nicotinic acid (or nicotinamide) than N′-methylnicotinamide, and no detectable amount of 6-pyridone. Following ingestion of nicotinamide, a great increase (20 to 100 fold) in nicotinic acid excretion occurred, but only a slight increase in N′-methylnicotinamide. Determinations of 6-pyridone were made by a newly developed method [Rosen *et al.* (236)] in which the pyridone is separated from N′-methylnicotinamide by extraction into ether and transformed into a fluorescent substance of unknown structure by shaking with acetone and potassium hydroxide.

Singal *et al.* (267) independently found that the addition of threonine to the diet of rats fed a low protein diet produced growth retardation which was corrected by either tryptophane or nicotinic acid. This agrees with a report of Hankes *et al.* (107), who showed that phenylalanine had a similar effect. In the diet used (267), threonine was actually the limiting essential amino acid, but its growth-promoting action was observable only when its inhibitory action was counteracted by nicotinic acid. Assay of tissues of rats rendered nicotinic acid-deficient by a low protein-corn grits ration showed that normal nicotinic acid levels were maintained in heart, spleen, lung, kidney, and blood; liver, brain, and muscle showed a subnormal content of nicotinic acid [Singal *et al.* (268)]. Liver storage of nicotinic acid on tryptophane supplemented diets was uniformly higher than on nicotinic acid supplemented diets (107).

Singal *et al.* (266) found that 0.1 per cent of added DL-tryptophane fully protected puppies against blacktongue. Only L-tryptophane was utilized as a precursor of nicotinic acid. Additional casein sufficient to supply 0.1 per cent of tryptophane failed to protect, although 42 per cent of added casein ($\approx$0.5 per cent of L-tryptophane) fully protected the animals. Thus, tryptophane added alone is much more effective than when added with protein. A similar result was obtained by Olcese *et al.* (210) in the rabbit, and by Rosen & Perlzweig (235) in rats. The latter workers found that the amount of N′-methylnicotinamide excreted by rats was much less when tryptophane was administered with gelatin than when it was administered alone. Such a difference was not observed when nicotinic acid was given, consequently these authors believe the growth-depressing effect of gelatin (or of the growth-depressing amino acids contained therein) on nicotinic acid low diets, results from an inhibition of the conversion of tryptophane to nicotinic acid, rather than from induction of an increased requirement for nicotinic acid.

To fulfill its rôle in protein synthesis, tryptophane must be fed together with other essential amino acids; this is not necessary to permit its utilization for synthesis of nicotinic acid [Geiger *et al.* (89)]. This synthesis cannot be carried out by *Tetrahymena geleii*, which requires both tryptophane and nicotinic acid for growth [Kidder *et al.* (158)].

Sarett & Goldsmith (239) reported results of an extensive study on the role of tryptophane as a nicotinic acid precursor in human beings. Here too, excretion of N′-methylnicotinamide and the acid-labile precursor of nico-

tinic acid [quinolinic acid (117)] was greatly increased by administration of tryptophane. The amounts excreted were not changed by administration of vitamin B_6. Frazier *et al.* (81) report a statistically significant increase in the amount of N'-methyl-nicotinamide excreted by women during the last five months of pregnancy.

Insulin-induced hypoglycemia in the chick embryo was reduced by injection of nicotinamide in experiments of Zwilling (358). Claims that injection of nicotinamide into children or adults produced a regular fall in the level of blood sugar could not be confirmed by Banerjee & Ghosh (6).

The fall in nicotine observed when cigar tobacco is fermented is accompanied by a marked rise in nicotinic acid. Up to 0.4 per cent of nicotinic acid could be isolated from the dried fermented leaves [Frankenburg & Gottscho (77)].

VITAMIN B_6

For the past several years, nomenclature of the naturally occurring compounds with vitamin B_6 activity has been ambiguous because of a widespread tendency to use "pyridoxine" both as a general term to include all naturally-occurring compounds with vitamin B_6 activity (including pyridoxal and pyridoxamine) and as the name for the specific compound, 2-methyl-3-hydroxy-4,5 bis (hydroxymethyl) pyridine. Correct practice has now been defined by the adoption of a report of the Committee on Biochemical Nomenclature of the American Society of Biological Chemists. In accord with common practice, the name *pyridoxal* has been adopted for the compound, 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine, and the name *pyridoxamine* for the compound, 2-methyl-3-hydroxy-4-amino methyl-5-hydroxymethylpyridine. The report reads further:

> It is further recommended that the term vitamin B_6 be used as a group name to include pyridoxal, pyridoxamine and pyridoxine, and that these specific names be used only where the corresponding chemical compound is meant. This recommendation specifically recognizes that the terms "vitamin B_6" and "pyridoxine" should no longer be used synonymously.

Little information is yet available on the distribution of the three forms of vitamin B_6 in natural products. Rabinowitz & Snell (223) utilized improved microbiological and extraction procedures to make such determinations. Pyridoxal and pyridoxamine were the predominant forms of vitamin B_6 in hydrolyzed animal tissues and yeasts. Little if any pyridoxine was found in these products. In plant materials, however, pyridoxine occurred commonly together with pyridoxal and pyridoxamine. The estimation of pyridoxine by such procedures is not entirely satisfactory, since it depends upon the subtraction of the sum of the figures found for pyridoxal and pyridoxamine from that found for total vitamin B_6. Where pyridoxine represents only a small fraction of the total vitamin B_6, as is frequently the case, it cannot be estimated satisfactorily by any known method.

These results indicate that on natural diets, most of the vitamin B_6 ingested is present as pyridoxal and pyridoxamine. To compare the metabolic

disposition of these substances and pyridoxine, Rabinowitz & Snell (224) administered 100 mg. of each compound to individual human adults and measured excretion of 4-pyridoxic acid, pyridoxal, pyridoxamine, and pyridoxine. The only form of the vitamin excreted in response to feeding all three forms of vitamin B_6 was pyridoxal. The chief product found in every case was 4-pyridoxic acid, and pyridoxal gave rise to significantly higher amounts of this product than did pyridoxamine or pyridoxine. Only 40 to 80 per cent of the ingested vitamin could be recovered as known excretion products.

Objective evidence of a requirement for vitamin B_6 by human beings has come from two groups. Greenberg *et al.* (97) noted a marked increase in xanthurenic acid excretion in response to a test dose of tryptophane in two human subjects after two to three weeks on a vitamin B_6-deficient diet. This increase was eliminated by administration of 15 mg. per day of pyridoxine hydrocholoride over a one week period. Such a derangement in tryptophane metabolism is one of the earliest symptoms of vitamin B_6 deficiency in other higher animals so far investigated.

McGanity *et al.* (188) found that pregnant women suffering from nausea and vomiting (*hyperemesis gravidarum*) showed a significantly lowered blood urea level as compared to normal pregnant women. Such patients also showed an abnormal change in blood urea following administration of a test load of DL-alanine. Administration of pyridoxine hydrochloride (40 mg. per day for three days) resulted in a change to the norm for healthy pregnant women in both cases. Although the low blood urea found in these patients is opposite to the condition found in vitamin B_6-deficient rats, the response to a test load of DL-alanine was entirely similar, and was shifted in a similar manner by administration of the vitamin.

Rhesus monkeys fed a vitamin B_6-deficient diet begin to eat less and lose weight slowly after 2 to 3 weeks. Gradual weight loss continues for six to nine months with few changes in external appearance. With longer periods of deficiency, the animals become unkempt, sluggish but hyper-irritable, and show changes in the hair coat. The most constant and prominent pathological finding encountered in such deficient animals [Rinehart & Greenberg (232)] was sclerotic lesions in the arteries of the pancreas, kidney, heart and elsewhere. The fibrous tissue plaques formed resembled closely those found in human arteriosclerosis. The authors emphasize that vitamin B_6 deficiency is chronic in nature, relatively slow in onset, and without distinctive external manifestations, and consequently would be particularly difficult to recognize clinically. The vitamin B_6 levels in the blood fell from 5 to 25 μg. per 100 cc. of blood (ave. 11 μg.) to 2 to 3 μg. (expressed as pyridoxine hydrochloride), or even lower within two to four weeks after starting on the vitamin-deficient ration [Greenberg & Rinehart (98)], then remained relatively constant throughout the course of the deficiency.

Vitamin B_6 deficiency in the Syrian hamster is characterized by muscular weakness, loss of luster and thinning of the hair coat, failure to grow, and the early appearance of increased xanthurenic acid in the urine [Shwartzman & Strauss (262)]. Death occurs after 12 to 13 weeks. Necropsy showed loss

of fat tissue and atrophy of lymphatic glands. Animals were rapidly restored to health by administration of pyridoxine following nine weeks on the deficient ration. Although administration of corn oil delayed the onset of deficiency symptoms, its administration appeared of no benefit once a deficiency was established.

Because the high serum iron and hemosiderosis of liver and spleen, characteristic of vitamin B_6 deficiency in swine, were not observed on an iron-low ration, Gubler *et al.* (103) determined the effect of vitamin B_6-deficiency in rats on the absorption of iron. After 12 weeks on the deficient and control rations, animals were killed, ashed, and total body iron and copper determined. Both mineral elements were significantly higher in the vitamin B_6-deficient animals, indicative of increased absorption by the deficient animals. Bowles *et al.* (18) report development of corneal vascularization in vitamin B_6-deficient rats provided the animals are 45 to 57 days of age before being placed on the deficient ration. Younger rats die of vitamin B_6 deficiency before this symptom develops.

Injection of 4-desoxypyridoxine into eggs results in death of the embryos at three to six days of age [Cravens & Snell (50)]. This toxic effect is prevented by simultaneous injection of either pyridoxal, pyridoxamine, or pyridoxine, a result which demonstrates that the inhibitor acts here, as in other organisms, by preventing utilization of vitamin B_6, and that vitamin B_6 is essential for early embryonic development. The ratio of vitamin to inhibitor which permitted approximately 50 per cent of the embryos to survive and hatch was 1/20 for pyridoxal, 1/50 for pyridoxamine, and 1/100 for pyridoxine. The inhibitor became relatively less effective when injected at four or more days of incubation (50). Wooley & Murphy (349) showed that desoxypyridoxine would inhibit multiplication of the T_2 bacteriophage of *E. coli* without inhibiting growth of the host bacterium. This effect of desoxypyridoxine was nullified by small amounts of pyridoxine, or by larger amounts of lower fatty acids (e.g., acetic, valeric, caproic acids) or of certain intermediates of carbohydrate metabolism (e.g., glucose-6-phosphate, pyruvic acid). In contrast is the observation [Lefwich *et al.* (169)] that the susceptibility of mice to pneumonia virus is decreased when the mice are fed either 4-desoxypyridoxine or diets deficient in vitamin B_6. Umbreit & Waddell (314) have shown that desoxypyridoxine inhibits neither the formation of pyridoxal phosphate nor the action of tyrosine decarboxylase in *Streptococcus faecalis*. The vitamin analogue is, however, phosphorylated by this organism to yield desoxypyridoxine phosphate, which inhibits the action of tyrosine decarboxylase by interfering with the combination of this enzyme with its natural coenzyme, pyridoxal phosphate. This provides the first known example of an antivitamin which is built into an analogue of a coenzyme before exerting its inhibitory effects.

For *T. geleii*, pyridoxal and pyridoxamine are equally as effective in promoting growth, and are about 1,000 times as active as pyridoxine [Kidder & Dewey (155, 156)]. In this respect *Tetrahymena* resembles bacteria such as *S. faecalis* (215) rather than the higher animals, which utilize all

three forms of vitamin B_6 with almost equal effectiveness (240). As with *S. faecalis*, it is probable that pyridoxine would show no activity if its conversion to pyridoxal or pyridoxamine through interaction with other components of the medium could be avoided (274). A previous erroneous conclusion to the effect that *Tetrahymena* did not require vitamin B_6 [Kidder & Dewey (157)] is now ascribed (156) to the fact that *Lactobacillus casei*, used by them to show that their early medium was free of vitamin B_6, does not measure all of the forms of vitamin B_6 present. On these grounds, they emphasize the unsuitability of *L. casei* for "pyridoxine" assays. It had been demonstrated much earlier (274), however, that *L. casei* was unsuitable for assay of "pyridoxine" (vitamin B_6). This is now known to result from the fact that of the three known forms of this vitamin, only pyridoxal has activity for *L. casei*, a fact which has been of great value in permitting estimation of this form of vitamin B_6 (215).

Umbreit & Gunsalus (313) have demonstrated conclusively that codecarboxylase is not identical with pyridoxal-3-phosphate, as again claimed by Karrer *et al.* (149). Samples of the latter obtained from Karrer were less than 1/1,000 as active as preparations of the pyridoxal phosphate of unspecified structure (presumably pyridoxal-5-phosphate) in stimulating decarboxylation of tyrosine by tyrosine decarboxylase.

Braunshstein *et al.* (20) showed that pyridoxal phosphate was the prosthetic group of an enzyme, kynureninase, present in the liver of all animals investigated, which degrades kynurenine to anthranilic acid and alanine. The livers of vitamin B_6-deficient rats contained only about one-third as much kynureninase as did livers of normal animals. If a similar enzyme is required for degradation of 3-hydroxykynurenine to 3-hydroxyanthranilic acid, the role of vitamin B_6 in the conversion of tryptophane to nicotinic acid (290) would be at least partially explained.

Broquist & Snell (24) showed that *L. arabinosus* apparently synthesized histidine from purine bases, and that vitamin B_6 was required for the transformation. *S. faecalis* and *L. arabinosus* could utilize imidazole pyruvic acid in place of histidine when vitamin B_6 was present, but not when this vitamin was absent. Vitamin B_6 is thus apparently involved in the transformation of certain keto acids, other than oxaloacetic and α-ketoglutaric acids, to the corresponding amino acids.

Several years ago, it was observed that D-alanine would replace vitamin B_6 as a growth essential for *S. faecalis* or *L. casei* in otherwise complete media [Snell (273)]. It was considered possible that D-alanine was serving as a precursor from which the cells synthesized the vitamin. Analysis of the vitamin B_6 content of cells of these bacteria grown with D-alanine showed, however, that little if any vitamin B_6 was synthesized [Holden *et al.* (137)]. Cells grown with limiting amounts of vitamin B_6 contained much more of the vitamin than did those grown with D-alanine; those grown with an excess of vitamin B_6 contained still more of the vitamin. Cells grown with vitamin B_6 but without D-alanine, however, proved to contain about 2 per cent of D-alanine, as they did when grown with this amino acid and without vitamin

B_6 [Holden & Snell (198)]. Thus D-alanine must either be supplied preformed, or must be synthesized by the cells to permit growth to occur; and synthesis of this essential D-amino acid requires vitamin B_6. Vitamin B_6 was postulated to act as a coracemase in the synthesis of D- from L-alanine.

A further reflection of the role of vitamin B_6 in metabolism of amino acids is provided by the observation (Bartlett & Gaebler (8)] that vitamin B_6-deficient dogs show a decreased ability to convert protein to carbohydrate, as indicated by a decreased dextrose:nitrogen ratio in the phlorizined animal on a high protein ration. The ratio was returned to normal by administration of pyridoxine hydrochloride. No such impairment was noted during deficiencies in thiamine or riboflavin.

The intracellular distribution of vitamin B_6 in livers of the mouse and rat was determined by Price *et al.* (222). Twenty-eight to forty-five per cent of the total vitamin was concentrated in the washed large granules (mitochondria); most of the remainder was in the supernatant liquid with only small amounts in the washed nuclear fractions and in the washed small granules.

Pantothenic Acid

Several reports of previously unrecognized or little emphasized symptoms which result from pantothenic acid deficiency in animals have appeared. Bowles *et al.* (18) showed that when rats well past weaning age are maintained on pantothenic acid-deficient rations for prolonged periods of time, many of them develop a pronounced corneal vascularization, characterized by numerous large capillaries with prominent anastomoses. Clouding of the cornea also occurs. These symptoms are rapidly eliminated by supplementation of the diet with pantothenic acid. Young rats fed the same diet died of the deficiency before developing the corneal symptoms. Under somewhat similar conditions, Berg *et al.* (9) noted that their rats, in addition to the usual symptoms of pantothenic acid deficiency, showed atrophy of the duodenal mucosa, while 60 per cent of them showed one or more well-developed duodenal ulcers. These ulcers resembled the so-called acute and chronic ulcers of human beings both microscopically and macroscopically. Parts of the gastrointestinal tract other than the duodenum were unaffected. Ziskin *et al.* (357) report necrotic ulcers of the tongue as occurring in pantothenic acid-deficient rats. Levy (170) gives details of several changes in bone and associated structures which occur in pantothenic acid-deficient mice.

Dumm *et al.* (58) confirmed results of previous workers in showing that application of stress (prolonged swimming) produced a marked lymphopenia in rats similar to that induced by injections of adrenocorticotrophic hormone (ACTH). In pantothenic acid-deficient rats this response to swimming or to injections of ACTH was partially abolished, and was restored by administration of the vitamin. The authors believe these results reflect the changes induced by pantothenic acid deficiency in the adrenal cortex—a tissue long known to be especially sensitive to deficiency of this vitamin.

Massive doses (12 to 20 gm.) of calcium pantothenate had no effect on

levels of blood glucose or of blood phosphate in human patients suffering from Addison's disease, diabetes, or cirrhosis of the liver. Administration of glucose or of insulin did not alter the excretion of pantothenic acid, which amounted to 9 to 15 per cent of the administered dose of calcium pantothenate, and as much as 60 per cent of the ingested pantothenol [Gershberg *et al.* (91)].

Hegsted & Riggs (114) redetermined the pantothenic acid requirement of chicks grown on a "synthetic" ration. This was found to be 900 to 1,000 μg. per 100 gm. of ration, a figure very close to the 1,100 μg. per 100 gm. previously found for ducklings (113). The authors view these requirements as minimal. On practical rations, somewhat more pantothenic acid might be required, since most of the vitamin would then be present as coenzyme A, which is only about 60 per cent as active for chicks, when fed orally, as would be predicted from its pantothenic acid content (112). On the molar basis, pantothenol was 86 per cent as active as pantothenic acid in supporting growth of chicks [Hegsted (111)].

The discovery that coenzyme A contains pantothenic acid and is necessary for acetylations has stimulated several investigators to determine the effect of pantothenic acid deficiency on the ability of the rat to carry out acetylation reactions. Shils *et al.* (259) injected sulfanilamide intraperitoneally into control and pantothenic acid-deficient rats and analyzed for free and acetylated sulfanilamide in the urine. Significantly lowered percentages of acetylsulfanilamide were excreted by the deficient animals. The same result was obtained when control and deficient groups were pair-fed. Addition of pantothenic acid to the ration rapidly restored the capacity for acetylation. No difference is acetylation capacity of control and thiamine-deficient rats was observed. Riggs & Hegsted (231) found, however, that although the capacity of rats to acetylate *p*-aminobenzoic acid is reduced most dramatically in pantothenic acid deficiency, it is also reduced in riboflavin deficiency and, over a limited range of concentrations of *p*-aminobenzoic acid, in thiamine deficiency. Neither excess calcium pantothenate nor acetate raised the acetylation capacity of riboflavin-deficient rats.

The finding that coenzyme A is essential for the enzymatic condensation of acetate and oxaloacetate to yield citrate in cell-free systems [Stern & Ochoa (285)] has its counterpart in the observation that homogenized heart ventricle from pantothenic acid-deficient ducklings forms citrate at a considerably lower rate than the corresponding tissues from normal ducklings [Olson *et al.* (211)]. Additions of coenzyme A stimulated citrate formation in both homogenates.

Novelli *et al.* (205) have described in detail their studies on the liberation of pantothenic acid from coenzyme A. This requires two separate enzyme preparations; an intestinal phosphatase, and an extract of acetone-dried pigeon liver. Each enzyme destroys the coenzymatic activity of coenzyme A, through degradation to products of unknown structure. Intestinal phosphatase liberates inorganic phosphate from the coenzyme; the product (impure) appears to be about 1/3 as active for *L. arabinosus* as an equivalent

amount of pantothenic acid. Assays of the same tissue extracts by enzymatic and microbiological methods indicate that essentially all of the pantothenic acid of most tissues is present as coenzyme A. King *et al.* (159), however, have described the occurrence and purification of a conjugated form of pantothenic acid which is devoid of coacetylase activity and which is more active than an equivalent amount of pantothenic acid in promoting growth of *Acetobacter suboxydans*. Concentrates of this conjugate are high in glutamic acid, and appear less readily dialyzable than coenzyme A. Novelli *et al.* (204) have shown that coenzyme A and the cleavage product formed from it by pigeon liver enzyme have this same type of enhanced activity for *A. suboxydans*, and suggest that the conjugates of King *et al.* (159) may be this degradation product of coenzyme A, which also is devoid of coacetylase activity. The cleavage product formed from coenzyme A by intestinal phosphatase is no more active for *A. suboxydans* than would be predicted from its content of pantothenic acid. Harrison (108) showed that treatment of natural materials with cold alkali (10*N*, room temperature, 5 min.) markedly increases the free pantothenic acid content, as indicated by assay with *L. arabinosus*.

Lipton & Strong (176) describe attempts to synthesize the keto analogue of pantothenic acid. The ketolactone corresponding to pantoyl lactone is readily obtained; condensation of this with β-alanine was, however, extraordinarily difficult to accomplish. A product which contained 13 to 15 per cent of the keto analogue (as indicated by catalytic reduction to pantothenic acid) was obtained, and was wholly inactive in promoting growth of *L. arabinosus*.

An interesting sidelight on the nature of the requirement of certain mutant cultures of *Neurospora* for pantothenic acid is afforded by studies of Wagner (323). The living mutant is known to synthesize pantoyl lactone and β-alanine, but does not couple them to yield pantothenic acid. Extracts of acetone-dried mycelium, however, contain an enzyme system which effects this coupling to the same extent as that found in the parent *Neurospora* culture, which does not require pantothenic acid for growth. The requirement of this "pantothenicless" *Neurospora* mutant for pantothenic acid thus does not result from lack of the necessary enzymes for synthesis of pantothenic acid, but is apparently due to the fact that action of these synthetic enzymes is in some way inhibited in the living mutant culture, but not in its parent strain. It appears possible that some instances of adaptive enzyme "formation" may result from the converse of this phenomenon.

Biotin

As early as 1943 Burk & Winzler (30) postulated that biotin functions in carbon dioxide transfer through the reversible opening and closing of its ureido ring. To examine this postulate, Melville, Pierce & Partridge (190) synthesized biotin with C^{14} as the ureido carbon atom and used the compound as a source of biotin in the growth of *L. arabinosus* on an aspartic acid-free medium. Under these conditions, as demonstrated by Lardy *et al.*

(167), biotin is required for the fixation of carbon dioxide in aspartic acid by this organism. Following growth, aspartic acid was isolated from the cells and found to be devoid of radioactivity. Similarly, the biotin isolated after dilution with inactive biotin from cells and medium accounted for all of the original radioactivity. These experiments indicate that reversible opening and closing of the ureido ring of biotin does not occur during biotin-catalyzed carbon dioxide fixation.

Although biotin has been implicated in the formation and decarboxylation of oxaloacetate, Plaut & Lardy found (220) that purified preparations of the oxaloacetate decarboxylase of *Azotobacter vinelandii* were nearly devoid of biotin. The biotin content of less pure preparations did not correlate with their decarboxylase activity. These findings confirm those of Ochoa *et al.* (206) with preparations of oxaloacetate decarboxylase from animal tissue.

The ability of cells of *L. arabinosus* to fix radioactive carbon dioxide was a direct function of the biotin content of the medium in which the cells were grown [Lardy *et al.* (167)]. Aspartic acid isolated from the cells was radioactive. Antimetabolites of biotin (e.g. homobiotin) inhibited the fixation of carbon dioxide by low-biotin cells in the presence of added biotin, but had little effect on the capacity of high-biotin cells to fix carbon dioxide. Aspartic acid itself inhibited the fixation of carbon dioxide by cells of *L. arabinosus.*

MacLeod & Lardy injected C^{14}-labeled bicarbonate into normal and biotin-deficient rats and compared the C^{14} content of the adenine, guanine, arginine, aspartic acid, citric acid, and bone carbonate subsequently obtained from tissues of the two sets of animals (181). In each case, more C^{14} was present in compounds from the normal animals, again emphasizing the role of biotin in carbon dioxide fixation.

The ability of biotin to restore deaminase activity to bacterial cells that have been aged or exposed to molar phosphate buffer at pH 4, originally described by Lichstein & Umbreit (174) and challenged by Axelrod *et al.* (5), has been confirmed with reservations by Wright *et al.* (352). In continued work on the problem, Lichstein reported (172) that yeast extract is about 100 times more active in restoring deaminase activity to treated cells than can be accounted for on the basis of its content of free biotin. These data were interpreted to indicate the presence in yeast extract of a coenzyme form of biotin more active than biotin itself in the system studied or, alternatively, the existence of forms of biotin undeterminable by microbiological assay. Preliminary results (173) favored the former hypothesis.

In 1944 Wright & Skeggs (351) showed that if the biotin content of yeast extract and certain other natural materials were determined microbiologically without prior acid hydrolysis, a much lower value was obtained with *L. arabinosus* than with *L. casei.* Acid hydrolysis did not alter the biotin content as determined by *L. casei* but increased the value obtained with *L. arabinosus* to that given by *L. casei.* These data indicated the existence of a form of biotin available to *L. casei* but not to *L. arabinosus.* Bowden & Peterson

confirmed and extended these findings (17), and showed that this form of biotin, termed soluble-bound biotin, differs from biotin in solubility. Soluble-bound biotin is resistant to a variety of enzymes but yields biotin or its microbiological equivalent on acid hydrolysis.

The combined form of biotin originally described by Wright & Skeggs (351) has been termed "biocytin" (Gr. *Kytos*, cell) and its isolation now has been accomplished (353). From several tons of yeast extract a few milligrams of crystalline biocytin were isolated. Recrystallized biocytin melts with decomposition at 203° to 210° C. and yields about 40 per cent of biotin or its microbiological equivalent on acid hydrolysis.

Further evidence for a metabolic relationship between biotin and fatty acids has accumulated. Trager (307) studied the ability of the fat-soluble factor from hydrolyzed horse plasma to replace the biotin requirement of chicks. Although the fat-soluble factor was originally reported (309) to have biotin activity in chicks, the data presented (307) indicate that such activity is restricted to a slight amelioration of the dermatitis characteristic of biotin deficiency in this species. Oleic acid was inactive against the dermatitis, and like the fat-soluble factor did not promote growth.

The ability of the fat-soluble factor, pimelic acid, oleic acid, lecithin, Tween 80, and Tween 40 to replace biotin as a growth factor for the larvae of *Aedes aegypti* has been studied by Trager (308). All of these substances, except pimelic acid, replaced biotin in promoting growth and metamorphosis of the larvae.

In continued studies on lipid stimulation of *L. casei*, Williams & Fieger (336) showed that rice polish lipids, which replace biotin for *L. casei* under appropriate, defined conditions, were inactive in substituting for biotin in the nutrition of the chick. The lag period observed when *L. casei* is grown with oleic acid in lieu of biotin could be shortened by the addition of sterile, biotin-free, bovine serum albumin to growth media. In fact, the rate of growth then exceeded that obtained with biotin but it is not clear whether or not this rate of growth can be attributed to unrelated stimulants present in the albumin used. Surface active compounds studied, that did not contain unsaturated fatty acids, were without activity in replacing biotin for *L. casei*.

In sufficient amounts, biotin produced effects on the electrophoretic mobility of cells of *L. casei* similar to those produced by cationic detergents, and on this basis it was postulated that biotin functions in *L. casei* because of its surface activity, and that similar surface activity explains the ability of oleic acid to eliminate the requirement for biotin [Williams & Fieger (337)]. An alternative explanation for this nutritional equivalence of biotin and oleic acid assumes that biotin is essential for synthesis of the unsaturated fatty acids; the addition of these products renders this synthesis unnecessary, and thus greatly reduces or entirely eliminates the requirement for biotin [Williams *et al.* (338)].

In contrast to the lactobacilli, *Neurospora crassa cholineless* cannot utilize oleic acid, aspartic acid, or Tween 80, alone or in combination, in place of

biotin and these products stimulated growth of this organism only slightly, even in the presence of biotin [Hodson (123).

Further details of the metabolic behavior of oxybiotin and its analogues have been supplied by Axelrod, Hofmann and co-workers. Paralleling the growth-promoting effect of oxybiotin in biotin-deficient chicks, there is a simultaneous increase in the oxybiotin content of liver, heart, spleen, lung, and leg muscle (187). The biotin content of the tissues was unaffected by administration of oxybiotin and remained at the level observed in the biotin-deficient chicks. Oxybiotin, like biotin, is incorporated into "bound" forms in tissues. Balance studies further demonstrated (186) that oxybiotin is utilized by the chick as such.

The antagonistic action of many oxybiotin analogues has been summarized (4). These analogues are more effective antagonists of oxybiotin than of biotin. Homooxybiotin, one of the more effective antagonists according to *in vitro* tests, was ineffective in protecting mice against experimental infection with *Streptococcus hemolyticus*.

An uncomplicated biotin deficiency is produced in the mouse by feeding diets containing egg white and sulfaguanidine. Symptoms were similar to those previously seen in biotin-deficient rats. Rusting of the fur and diarrhoea were also observed [Wilson *et al.* (343)].

Carlson & Carlson (38) report that three strains of *Leuconostoc* did not require biotin for growth in a sucrose medium although the same organisms did require the vitamin for growth in a glucose medium. The result apparently was not due to the presence of biotin in the sucrose as a contaminant.

Folic Acid (Pteroylglutamic Acid, *L. casei* Factor)

Chemistry.—Syntheses of 9-methylpteroylglutamic acid and derivatives (134), 4-aminopteroylglutamic acid and derivatives (257), and derivatives in which the glutamic acid of pteroylglutamic acid or 4-aminopteroylglutamic acid (358) is replaced by other amino acids (258) have been described.

Pteroyl-γ-glutamylglutamic acid and pteroyl-γ-glutamyl-γ-glutamylglutamic acid were synthesized by Boothe *et al.* (15) by improved methods. The latter had the same microbiological activity and infrared absorption spectra as the fermentation *L. casei* factor. All of the five possible isomers pteroylglutamylglutamylglutamic acid now have been synthesized, and it is possible to state conclusively that the fermentation *L. casei* factor is pteroyl-γ-glutamyl-γ-glutamylglutamic acid.

N^{10}-nitrosopteroylglutamic acid, prepared by Cosulich & Smith (48) from the reaction of pteroylglutamic acid with nitrous acid, equals pteroylglutamic acid in growth-promoting activity for *S. faecalis* R and the chick.

A series of 2, 4-diamino-6,7-diphenylpteridines were prepared by Cain *et al.* (35) and their activities as antagonists of folic acid in the growth of *S. faecalis* R determined. Certain of them were highly active antagonists.

In a careful study of the action of ultraviolet light on folic acid, Lowry *et al.* (177) demonstrated the successive formation of 2-amino-4-hydroxy-6-formylpteridine (xanthopterin aldehyde), 2-amino-4-hydroxy-6-carboxy-

pteridine, and finally by decarboxylation 2-amino-4-hydroxypteridine. This latter compound is oxidized by xanthopterin (xanthine) oxidase to 2-amino-4, 7-dihydroxypteridine (isoxanthopterin). Various problems connected with the fluorometric estimation of folic acid have been studied (1, 2, 317).

Microbiology.—Hodson (122) applied various procedures to the determination of folic acid in milk and concluded that none of the presently available methods yielded accurate results with this product. However, milk was a poor source of folic acid, whether values were determined by chick or microbiological assay.

Sreenivasan *et al.* (284) compared the utility of various conjugase preparations for liberation of folic acid from natural materials. Their data further emphasize that no one procedure is applicable for the release of "combined" folic acid from all materials. The successive use of conjugases from various sources and the use of such preparations at various pH ranges were suggested as methods likely to yield maximum values for folic acid.

Animal nutrition.—Hematologic and histologic aspects of folic acid deficiency in the mouse have been studied by Weir *et al.* (327). A reduction in the cellular elements of the circulating blood and arrest of maturation in the bone marrow with hyperplasia of immature elements and reduction of adult forms were observed. These findings were taken as evidence for the involvement of folic acid in maturation of primitive elements rather than in the formation of these elements.

Williamson (340) and Nelson & Evans (196) have emphasized the high requirement of the rat for folic acid during pregnancy and lactation.

Addition of thymine and purine bases to a complete medium replaces folic acid for many lactic acid bacteria; the same mixture was not active in lieu of folic acid in the rat [Petering & Delor (215)]. Several groups had previously shown the inactivity of thymine alone; the mixture had not been previously tested in rats. A rat growth method for determination of folic acid in natural materials has been described by Asenjo (3).

Various aspects of folic acid deficiency in the rabbit (264), the pig (39, 40), duckling (193), turkey (249), mink and fox (304), 305) have been described.

Clinical.—Knott & Suarez observed (163) a slightly decreased urinary excretion of folic acid in patients with sprue as compared to that found in normal controls.

Teropterin (fermentation folic acid) has been reported to ease pain when administered to cancer patients. Slaughter studied (271) this reported analgesic effect in normal patients with the aid of the Wolff-Hardy-Goodell apparatus and concluded that teropterin possesses true analgesic activity.

Metabolism.—Buyze & Engel (32) reported that pteroylheptaglutamic acid is acted upon by normal human gastric juice to give another, as yet unidentified, conjugate. This change in pteroylheptaglutamic acid was not brought about by the gastric juice of the patient with pernicious anemia but did occur with the gastric juice from a patient with sprue.

Villela & Mello found (318) that subcutaneous administration to mice

of fairly large doses of folic acid resulted in an increase in the total weight and alkaline phosphatase of the kidneys. This increase in weight was not due to water retention but probably resulted from an accumulation of solids accompanying renal damage.

Folic acid has been shown by Dinning *et al.* (52) to counteract the growth inhibition and decreased prophyrin excretion accompanying the oral administration of a large amount of glycine to rats. According to Keith & Totter (152) and Totter *et al.* (303), folic acid also counteracts the growth inhibition and decreased fecal porphyrin excretion brought about by the administration of 3 per cent of sodium benzoate to rats.

Continued studies by Welch *et al.* (326) on the effect of incubating xanthopterin with liver show a consistently increased folic acid content (determined microbiologically) as a result of such incubation. The mechanism of the effect remains unexplained.

Simpson & Schweigert have observed (263) the occurrence of folic acid conjugases in the blood of several animal species. Blood conjugases release folic acid from the heptaglutamate at an optimum pH of 6 to 8. Wolff *et al.* (348) also demonstrated the occurrence of a folic acid conjugase in human blood which was not significantly lowered in cases of pernicious anemia. Hydrogen sulfide, cysteine, reduced glutathione and a dialyzable constituent of hemolyzed red cells increased the activity of the enzyme (347). Mercuric chloride, iodoacetic acid, cystine, oxidized glutathione, and calcium ions inhibited its activity. Conjugase preparations from chicken pancreas, in contrast to those from hog kidney, contain only one active component [Dabrowska, Kazenko & Laskowski (53)]. The enzyme is preferentially a γ-glutamic acid carboxypeptidase which requires at least three glutamic acid residues in the substrate for its activity.

Several papers indicate an intimate relationship between folic acid and riboflavin in metabolism. Daily oral administration of 10 mg. of folic acid to normal individuals is accompanied by marked decreases in the fecal excretion of riboflavin [Odland *et al.* (207)]. The administration of folic acid to rats on a purified diet results in a decreased D-amino acid oxidase content of the liver [Kelly (153), Williams *et al.* (334)]. Keith *et al.* (151) have reported that the xanthine oxidase activity of chick liver is inversely related to the folic acid content of the diet, and that this enzyme was present in greater amounts in livers of chicks fed a commercial diet than in chicks fed a purified diet plus folic acid. Westerfield & Richert (331), however, found that the xanthine oxidase activity of liver from rats fed purified diets was about one-half that seen in rats fed commercial diets, and postulated that a new dietary essential is necessary for the production of normal levels of liver xanthine oxidase. These latter findings were confirmed by Schmitt & Petering (247). Williams & Elvehjem (333) concluded that purified xanthine oxidase is inhibited by aldehyde-free folic acid, although much less strongly than by commercial material [cf. Kalckar & Klenow (146) with xanthopterin oxidase]. Similarly either commercial or aldehyde-free folic acid was reported to inhibit guanine oxidation and endogenous respiration of rat liver homogenates.

Hofstee (127) concluded that the enzymatic oxidation of xanthine and of xanthopterin is accomplished by a single enzyme. In agreement with Williams & Elvehjem, purified folic acid was found to inhibit the enzyme but the inhibition could be reduced to zero by preliminary incubation with an excess of the enzyme. A variety of pteridines had inhibitory effects on the enzyme. Leucopterin was relatively inactive as an inhibitor while pteroic acid or xanthopterin-6-aldehyde were among the most potent of the inhibitors. The kinetics of the inhibition of xanthopterin or xanthine oxidase by 2-amino-4-hydroxy-6-formylpteridine (xanthopterin aldehyde) have been studied by Lowry *et al.* (178).

Various reports have appeared confirming the essential nature of folic acid for the oxidation of aromatic amino acids and their metabolic products. Govan & Gordon (95) reported that administration of folic acid to premature infants decreases the urinary elimination of hydroxyphenyl derivatives. The ability of liver tissue from folic acid-deficient rats to oxidize tyrosine may be partially restored *in vitro* with folic acid but not by folic acid conjugate or liver extract [Rodney *et al.* (234)]. Liver from rats made folic acid-deficient by use of the antagonist, 4-amino-pteroylglutamic acid, could not be activated *in vitro* with folic acid, liver extract, or vitamin B_{12}, but could be activated by the *in vivo* administration of folic acid or liver extract. The addition of 4-aminopteroylglutamic acid to liver tissue from normal rats does not influence the rate of tyrosine oxidation.

Taylor & Carmichael (296) have reported that male mice tolerate subdermally injected doses of folic acid that would be lethal to female mice. The basis of this phenomenon was not determined. Folic acid has been claimed by Traina (310) to be an antianaphylactic agent.

Folic acid antagonists.—A large number of papers describe the use of folic acid antagonists as inhibitors of normal and tumor growth, bacterial multiplication, and enzymatic activity. It is beyond the scope of this review to discuss each paper individually. For purposes of summation Table I may be of value.

Vitamin B_{12}, Antipernicious Anemia Factor, Animal Protein Factor

Chemistry.—Ellis *et al.* described (65) the isolation of a crystalline antipernicious anemia factor from liver. Comparison with vitamin B_{12} in the Merck Laboratories (229) led to the conclusion that the two compounds were identical.

Reports from the Merck Laboratories (23) have described additional characterization studies on vitamin B_{12}. The compound contains 4.5 per cent of cobalt, indicative of a minimal molecular weight of 1,300. This value agrees well with an ebullioscopic determination, $1{,}490 \pm 150$. Elementary analysis indicates a composition of approximately $C_{61-64}\,H_{86-92}\,N_{14}\,O_{13}\,PCo$. The vitamin is a levo rotatory, polyacidic base, but the basic groups are quite weak. It shows absorption maxima at 2,780 Å, 3,610 Å, and at 5,500 Å that do not change markedly with pH, and does not contain a pterin-type structure. No amino acids could be identified by microbiological assay or by

TABLE I

FOLIC ACID ANTAGONISTS AS INHIBITORS OF VARIOUS TYPES OF BIOLOGICAL ACTIVITY

Antagonist	Species	Response studied	References
4-Amino folic acid	Bacteria	Growth	(80)
4-Amino folic acid	Frog	Oviduct response to estradiol	(94)
4-Amino folic acid and 4-Amino-N^{10}-methyl folic acid	Mouse	Sarcoma 180	(93, 194, 248, 302)
4-Amino folic acid and related compounds	Mouse	Transplanted leukemia	(26 to 29, 169, 328)
4-Amino folic acid	Mouse	Transplanted mammary tumors	(133)
4-Amino folic acid	Rat	Hormonal changes in prostate	(21)
2-Amino-4-hydroxy-6-formyl-pteridine	Rat	Oxygen consumption of brain *in vitro*	(45)
4-Amino folic acid	Rat and mouse	Toxicity	(121, 217)
4-Amino folic acid	Rat and mouse	Various tumors	(294)
4-Amino folic acid and related compounds	Rat and bacteria	Growth and hematopoesis	(295)
4-Amino folic acid, pteroyl aspartic acid, and 7-methyl pteroic acid	Guinea pig	Blood cell formation and bone marrow changes	(142)
Methyl folic acid	Chick	Response to testosterone propionate	(356)
4-Amino folic acid and related compounds	Chick	Growth of embryos	(148)
4-Aminopteroylaspartic acid, 4-Amino-N^{10}-methyl folic acid	Chick, rat, and bacteria	Growth	(78, 140)
4-Amino folic acid, 4-Amino-N^{10}-methyl folic acid, 4-Amino pteroylaspartic acid, and 2,6-Diamino purine	Chick, rat, and mouse	Growth, tissue culture, various tumors, embryos, blood cellular elements, and toxicity	(293)
4-Amino folic acid	Dog	Megaloblastosis	(300, 301)
4-Amino folic acid	Dog	Hemorrhage	(102)
X-Methyl folic acid	Dog	Folic acid deficiency	(79)
4-Amino-N^{10}-methyl folic acid	Dog, rat and mouse	Toxicity	(74, 218, 300)
2,6-Diamino purine	Dog, rat and mouse	Toxicity, blood cell elements	(211)
4-Amino folic acid	Human	Leukemia	(11, 51, 73, 139, 193, 324, 342, 346)
4-Amino folic acid and related compounds	Human	Various types of malignant disease	(90, 297)
4-Amino folic acid, 4-Amino-N^{10}-methyl pteroylglutamic acid, and 4-amino pteroylaspartic acid	Human	Bone marrow changes	(299)

paper chromatography in hydrolyzed preparations of the pure vitamin. Pyrolysis products of vitamin B_{12} gave a positive pine splinter test and a positive Ehrlich reaction, both indicative of a pyrrole or porphoryn-like structure. Acid hydrolysis of vitamin B_{12} yields a basic cleavage product identified as 5, 6-dimethylbenzimidazole [Brink & Folkers (22)]. Although this latter compound is similar to a portion of the riboflavin molecule, hydrolysis of riboflavin under similar conditions did not yield 5, 6-dimethylbenzimidazole.

By catalytic hydrogenation in the presence of platinum oxide, vitamin B_{12} gives rise to a new compound, vitamin B_{12a} [Kaczka *et al.* (145)]. The latter has essentially the same cobalt and phosphorus content as vitamin B_{12} and a very similar absorption spectrum. In preliminary tests it appeared less active than the parent compound for *Lactobacillus lactis*, *Lactobacillus leichmannii*, the rat, the chick, and the human patient with pernicious anemia. The possibility that vitamin B_{12a} may occur naturally was not discussed.

Smith (272), by chromatographic techniques, early described the existence of more than one pink clinically active pigment in liver extract. Pierce *et al.* (219) similarly noted two pink bands when fractions from *Streptomyces aureofaciens* were chromatographed on silicic acid. Crystalline material was isolated from each of the two bands. One product proved identical with vitamin B_{12}. The other contained cobalt and phosphorus and showed absorption maxima at 2,730 Å, 3,510 Å, and 5,250 Å. Maxima at 3,070 Å, and 3,250 Å, shown by vitamin B_{12}, were absent. Following the nomenclature suggested by the Merck Laboratories, the compound was tentatively named vitamin B_{12b}. Vitamin B_{12b} is active for *L. leichmannii* and the chick; quantitative comparisons with the activity of vitamin B_{12} have not been published.

Microbiology.—Under specified conditions, *L. lactis* Dorner requires vitamin B_{12} and was successfully used in the Merck Laboratories as an assay organism in studies leading to isolation of this vitamin. Subsequently, *L. leichmannii* 4797 [Skeggs *et al.* (270)], or 313, [Hoffmann *et al.* (124)] have been recommended for determination of this vitamin. Shive *et al.* (261), Kocher (320), Greene *et al.* (99), and Koditschek *et al.* (165) all find that under appropriate conditions the requirement of *L. lactis* for vitamin B_{12} is eliminated by ascorbic acid. The effect apparently results from induction in the medium of a sufficiently low oxidation-reduction potential, since other reducing agents, strict exclusion of oxygen, or prolonged autoclaving of the medium also permit growth in the absence of vitamin B_{12}. Welch & Wilson (325) reported that reducing agents (e.g., ascorbic acid, glucoascorbic acid, thioglycollic acid, glutathione, etc.) replaced vitamin B_{12} for *L. leichmannii* when the growth medium contained an enzymatic digest of casein, but not when this digest was absent. They suggest that inactive, oxidized forms of vitamin B_{12} may be present in such digests which are reduced to microbiologically active forms by these reducing agents. Similar findings were made by Kocher (320) with *L. lactis*. Hoff-Jørgensen (125) has also noted that a charcoal-treated tryptic digest of casein contains a factor capable of replacing vitamin B_{12} for certain bacteria.

Several authors [Shive *et al.* (260), Wright *et al.* (354), Snell *et al.* (276), and Kocher & Schindler (321)] found that thymidine also replaced vitamin B_{12} in the nutrition of several lactic acid bacteria, including *L. lactis* and *L. leichmannii*. Desoxyribosides of adenine, guanine, hypoxanthine, and cytosine are as effective in this respect as thymidine [Kitay *et al.* (162), Hoff-Jørgensen (126); Kocher & Schindler (321)]. Desoxyribonucleic acid is also utilized slowly in place of vitamin B_{12} by some of these organisms [Kitay

et al. (162)]. The activity of these six naturally-occurring compounds probably explains fully the results of Winsten & Eigen (344, 345) who demonstrated by partition chromatography on paper that refined liver extracts contained at least six entities which promoted growth of *L. leichmannii* in a medium free of vitamin B_{12}. Three of these were identified with thymidine, hypoxanthine desoxyriboside, and vitamin B_{12} (345).

This nonspecificity of lactic acid bacteria in their requirement for vitamin B_{12} complicates the use of these organisms for assay of this vitamin. Such interference is more serious when cup plate assays are used than when the more customary assays in liquid media are employed. In the former procedure vitamin B_{12} is only about ten times as active as thymidine in producing zones of growth; in the latter, vitamin B_{12} is about 10,000 times more active than thymidine [Skeggs *et al.* (269)]. Under the latter conditions, interference by the desoxyribosides frequently is negligible except in samples very low in vitamin B_{12}. Procedures for microbiological assay of vitamin B_{12} in liquid media are described by Caswell *et al.* (41), Hofman *et al.* (124), and Skeggs *et al.* (269, 270). A cup-plate procedure for assay with *L. lactis* in which the interference from desoxyribosides is said to be eliminated by addition of sodium chloride is described by Foster *et al.* (76).

Stokstad (289) has advocated the use of mild alkaline hydrolysis to destroy vitamin B_{12}, without concomitant inactivation of the desoxyribosides prior to differential microbiological assay of samples suspected or known to contain significant amounts of the desoxyribosides. Paper chromatography has also been widely used to separate the desoxyribosides from vitamin B_{12} prior to assay (344). Vitamin B_{12} may also be determined with *Euglena gracilis*, an organism which does not respond to thymidine [Hutner *et al.* (141)], but this procedure has not been extensively used.

While crystalline vitamin B_{12} is stable to autoclaving in bacteriological media, the vitamin B_{12} activity of crude products was reduced by this procedure [Stokstad *et al.* (291)]. Addition of thioglycollic acid to the basal medium prevented this inactivating effect of heat. Skeggs *et al.* (269) found thiomalic acid preferable to several other antioxidants, including thioglycollic acid, for this purpose.

Clinical.—Reports of the effectiveness of parenteral vitamin B_{12} against the hematologic and neurologic manifestations of pernicious anemia continue to appear (10, 71, 150, 191, 245, 281, 282, 329). The relative ineffectiveness of orally administered vitamin B_{12} in pernicious anemia has been emphasized by several groups. Thus, Berk *et al.* (10) observed that the daily oral administration of 5 μg. of vitamin B_{12} to four patients with pernicious anemia was without effect on the reticulocyte and erythrocyte counts. Daily administration of this amount of vitamin B_{12} together with 125 or 150 cc. of neutralized normal human gastric juice (inactive alone) to the same patients in successive periods produced reticulocytosis in all instances. In three of four patients, a significant rise in erythrocytes followed oral administration of vitamin B_{12} with normal human gastric juice. The authors conclude that the probable function of the intrinsic factor of normal human

gastric juice is to facilitate absorption of vitamin B_{12} rather than to react with an extrinsic factor as hitherto assumed. Rather similar results were obtained by Spies *et al.* (280) and by Hall, Morgan & Campbell (105). These latter workers also showed that the minimal daily amount of gastric juice required to potentiate an optimal hematopoietic response from a daily oral dose of 5 μg. of vitamin B_{12} lies somewhere between 25 and 150 cc.

A possible explanation for the effects of normal human gastric juice was advanced by Ternberg & Eakin (298), who report that normal gastric juice contains a protein, apoerythein, which combines specifically with vitamin B_{12} to form a complex termed erythein. Gastric juice from pernicious anemia patients contains greatly reduced amounts of apoerythein. The vitamin B_{12} of erythein is tightly bound, and is not available to microorganisms which require it. The vitamin is released by heat denaturation of the protein. Presumably erythein is either more readily absorbable than free vitamin B_{12}, or combination of the vitamin with the protein protects it from destruction and permits subsequent absorption.

Callender *et al.* (36) prepared an extract from the feces of a patient with untreated pernicious anemia that caused a reticulocyte response and an increase in hemoglobin when administered intramuscularly to a patient with pernicious anemia.

The parenteral administration of a single dose of thymidine to patients with pernicious anemia [5.3 mg., West and Reisner (330), 48 mg., Ungley (315), 150 mg., Reisner & West (228)], has been without beneficial effects, although Reisner & West did note a slight reticulocyte peak when thymidine alone (150 mg.) was given and a slight secondary reticulocyte peak when 5 mg. of thymidine was administered for nine days to a patient that was already receiving 0.25 μg. of vitamin B_{12} daily.

In addition to reports describing the effectiveness of vitamin B_{12} in pernicious anemia, there has appeared much corroborative evidence for the activity of vitamin B_{12} in the treatment of megaloblastic anemia of infancy (189), nutritional macrocytic anemia, sprue, and nutritional glossitis (279).

Animal nutrition: Mice.—Mice may be used in the assay of animal protein factor (vitamin B_{12}) by either of two methods [Bosshardt *et al.* (16)]. Depleted weanling rats from mothers maintained on a diet free of the animal protein factor respond well to additions of this vitamin to a purified diet. Alternatively, 0.5 per cent of thyroid may be fed with a synthetic diet to mice from "normal" mothers, and its growth-retarding effects counteracted by vitamin B_{12}. Crystalline vitamin B_{12} is active in both procedures, and a high positive correlation (0.89), in the results on a variety of samples by the two methods of assay was obtained. The results obtained on a series of samples by mouse assay are in agreement with those obtained by the *L. leichmanii* assay (270) when an adequate amount of reducing agent is incorporated in the microbiological assay medium (269). It was further demonstrated that the animal protein factor is readily transmitted from the mother to the young during gestation or lactation and may be stored by the mouse for a considerable period of time. A severe lack of the animal protein factor in the

maternal diet results in a pronounced mortality of young mice one to three days *post partum*.

Rats.—Crystalline vitamin B_{12} previously has been shown by Emerson *et al.* (66) to be active in promoting growth in rats. Additional reports describe the requirement for vitamin B_{12} for gestation and lactation and in the thyroid-fed rat. Emerson *et al.* (68, 69) have shown that the size and birth weight of litters cast by mothers fed vitamin B_{12} as a supplement to a basal soybean meal diet were no greater than the control litters. The weaning weights of the rats from mothers receiving vitamin B_{12} were 50 per cent greater than controls. When the young from each of the two groups were in turn divided at weaning into a control group and one supplemented with vitamin B_{12} the beneficial effects of vitamin B_{12} were further apparent.

Conflicting reports concerning the ability of vitamin B_{12} to counteract thyrotoxicity in rats have appeared. Emerson reported (67) that vitamin B_{12} counteracts the growth-retarding effect of thyroid powder when fed or administered parenterally in conjunction with a diet devoid of animal protein. Register *et al.* (226) similarly have obtained excellent growth responses (over short time intervals) to crystalline vitamin B_{12} administered intraperitoneally to rats fed iodinated casein. Betheil & Lardy reported (13) that crystalline vitamin B_{12} partially counteracted the growth retardation in rats fed thyroid substance. Ershoff (72) found that crystalline vitamin B_{12} was ineffective in promoting growth in rats fed desiccated thyroid. Differences in the rations exist and probably account for these discrepancies.

Stern *et al.* observed (286) that the basophilia (attributable to ribonucleic acid) of rat liver sections is decreased in the vitamin B_{12}-deficient rat. Thus it would appear that nucleoprotein (and protein synthesis that it regulates) is impaired in vitamin B_{12} deficiency. Hepatic ribonucleic acid decreases or disappears during carbon tetrachloride intoxication, and it has been shown by Popper *et al.* (221) that vitamin B_{12} has a protective effect in rats against hepatic injury produced by carbon tetrachloride.

Poultry.—Since the demonstration by Ott *et al.* (213) that crystalline vitamin B_{12} has "animal protein factor" activity in the chick, there have appeared a number of reports describing the rôle of vitamin B_{12} in the nutrition of the chick. Nichol *et al.* (199) reported that vitamin B_{12} administered orally or parenterally completely counteracts a thyrotoxic condition in chicks produced by feeding a basal ration containing 0.05 per cent of iodinated casein. Pure vitamin B_{12} replaced completely the animal protein factor activity of condensed fish solubles and injectable liver preparations.

Direct evidence that vitamin B_{12} is the factor involved in increasing the hatchability of eggs from hens fed plant protein diets has been obtained by Lillie *et al.* (175). When eggs obtained from such hens were injected with vitamin B_{12}, they showed improved hatchability and viability as compared with water-injected control eggs, and the chicken which hatched also showed improved growth and feathering.

Ott reported (212) that the minimum vitamin B_{12} requirement for maximum growth of chicks on a synthetic diet approximates 4 μg. per 100 gm. of

diet. Nichol *et al.* (199) found that at least 1.5 μg. of vitamin B_{12} per 100 gm. of diet is required for maximal rate of growth in the thyroid-fed chick.

Pigs.—Neumann *et al.* found (197) that vitamin B_{12} deficient pigs were irritable, sensitive to touch, and sluggish in their movements. Several such pigs were unable to stand on their rear legs. Hematopoiesis was deficient in contrast to the absence of an hematopoietic dyscrasia in the vitamin B_{12} deficient mouse, rat, and chick. Forty-two micrograms of vitamin B_{12} per kg. of dry matter in the vegetable protein diet used produced the maximum growth response. Administration of vitamin B_{12} or an equivalent amount (based on microbiological assay) of reticulogen led to equal growth responses [Johnson & Neumann (143)]. Hogan & Anderson (128) reported excellent weight gains in pigs given intramuscular injections of vitamin B_{12} to supplement a highly purified diet adequate in other known dietary essentials.

Burnside *et al.* report (31) that the value for pigs of peanut meal and soybean oil meal may be raised to equal that of fish meal by addition of vitamin B_{12}.

Distribution.—More study of extraction procedures, stability, and the specificity of response of various bacteria to vitamin B_{12} will be required before microbiological methods can be employed for the accurate assessment of vitamin B_{12} in anything other than high potency materials. A rat growth method for vitamin B_{12} was proposed by Register *et al.* (227), and applied to a study of the vitamin B_{12} content of various beef and pork samples (226) and of rat tissues [Lewis *et al.* (171)]. Beef contains more vitamin B_{12} than pork. The rat kidney was found to contain more vitamin B_{12} than any other tissue. Depletion of vitamin B_{12} in the liver, heart, small intestine, and muscle was observed in rats fed a ration low in vitamin B_{12} for six weeks. A similar assay procedure with rats has been studied by Frost *et al.* (83), who found approximately equal growth responses whether vitamin B_{12} was administered by mouth or by injection.

Choline

Dimethylthetin and its homologue, dimethyl-β-propiothetin, support growth and protect against hemorrhagic kidneys in rats fed a choline and methionine-free diet plus homocysteine; they are, therefore, excellent sources of labile methyl groups [Maw & du Vigneaud (185)]. Methylethylthetin showed some activity, diethylthetin and methylmercaptoacetic acid were wholly inactive. Sulfocholine (β-hydroxyethyldimethylsulfonium chloride), which bears the same relation to dimethylthetin that choline bears to betaine, protected rats against hemorrhagic kidneys but failed to support growth of rats, because of its toxicity. At low levels (0.2 per cent of the ration) it prevented the rapid weight loss characteristic in animals fed the test ration without supplementary labile methyl groups [Maw & du Vigneaud (184)]. This toxic action of sulfocholine could not be prevented by simultaneous administration of choline or dimethylthetin, hence the compound does not appear to act as an antivitamin. The compound is lipotropic in rats, an indication that it can serve in place of choline for phospholipid synthesis; treat-

ment of an isolated lipid fraction with sodium ethoxide yielded some dimethyl sulfide, in confirmation of this view (184).

Dubnoff & Borsook (57) showed that dimethylthetin and dimethyl-β-propiothetin permitted synthesis of methionine from homocysteine by homogenized liver preparations; dimethylthetin was 20 times more active than betaine in this respect. Dimethylethanolamine, dimethylglycine, and methylmercaptoacetic acid were inactive in this methylation, which, in the isolated system employed, was irreversible (57). Sulfocholine was also found by Dubnoff (56) to be highly active in this system; aerobic conditions were necessary, however, whereas dimethylthetin was active anaerobically. This suggests that sulfocholine acts in methylation only following oxidation to dimethylthetin. Inasmuch as previous data (184) indicated that sulfocholine did not promote growth under conditions requiring it to supply labile methyl groups, Dubnoff assumes that the compound is too toxic to be the sole source of methyl groups, in the diet. The existence of highly active enzymes in the livers of all animals tested which transfer methyl groups specifically from these sulfonium compounds suggests that these compounds play an important rôle in methylation reactions in biological systems. The isolation of dimethyl-β-propiothetin from the alga *Polysiphonia fastigiata* [Challenger & Simpson (42)] is in agreement with this point of view. If of general distribution, these compounds would constitute a previously unrecognized source of labile methyl groups in the diet.

Handler (106) found that seven different rations which produced fatty livers in rats failed to do so in guinea pigs; a fact which suggested that lack of choline oxidase in this species (in contrast to all other animal species investigated) results in a choline turnover so slow as materially to reduce the requirement for this compound by the guinea pig. Dubnoff (55) checked the comparative availabilities of choline and betaine for methylation reactions in the guinea pig by injecting these compounds labeled with C^{14}, and subsequently checking the respiratory carbon dioxide, proteins from liver, kidney and intestinal tract, and the methyl iodide derived from a quantitative methionine determination for radioactivity. By each test, betaine was 4.6 times more active as a methyl donor than choline; the results showed, however, that choline did act as a methyl donor in this species, despite the lack of choline oxidase in the liver.

Treadwell (311, 312) summarizes several relationships between the effects of dietary methionine, choline, and cystine on the growth of rats. Engel (70) noted that anemia consistently occurred when rats were held on a choline-low diet for three months or longer; in some animals this was accompanied by marked fluid accumulations in the abdominal cavity, and in the subcutaneous tissues. Neumann *et al.* (198) report that baby pigs require 0.1 per cent of choline on a diet which contains 30 per cent of casein. Choline deficiency in this animal results in a short-legged and pot-bellied appearance, general unthriftiness, lack of coordination in movements, and loss of proper rigidity of the joints, particularly in the shoulders. Fatty liver also occur. Noland & Baumann (202) found the German cockroach to require 2 to 4

mg. of choline per gm. of diet for optimum growth and maturation. This is the first report that choline is a nutritional essential for insects. Betaine replaced choline quantitatively at all levels of intake and led to deposition of choline in the tissues; ethanolamine was inactive.

A possible new rôle for choline under certain dietary conditions is emphasized by the finding that the methyl groups of choline serve as precursors from which the β-carbon atom of serine may arise, presumably via intermediate formation of formate [Sakami (238)].

Wick (332) found that pancreatic extracts prepared by the procedures of Eilert & Dragstedt (59) for preparation of lipocaic had a lipotropic action in rats which corresponded exactly to their choline content. A previously reported antagonism between heparin and choline could not be confirmed either *in vivo* or *in vitro* by Howe & Spurr (132). Schlegel (246) found the choline content of human serums to show a marked seasonal variation; the lowest values (0.3 per cent) occurred during July; the highest (1.5 per cent) during February and March. The cause of the variation is unknown, a plot of the values against time of year yielded a curve very similar to one showing the seasonal incidence of rachitis and tetany.

If growing rats are subjected to short periods (5 to 6 days) of choline deficiency—a period sufficient to yield hemorrhagic kidneys in many animals—and are then restored to a stock ration, a certain proportion of them subsequently develop hypertension, and show a persistence of renal lesions and an enlarged heart [Hartcroft & Best (109)]. The authors liken the changes which occur to those induced by a subtotal nephrectomy.

Vitamin B_{12} administration markedly reduces the amount of choline required to prevent hemorrhagic kidneys in rats and to permit optimal growth in the chick [Schaefer *et al.* (243, 244)]. Preliminary results of a similar nature were reported by Jukes & Stokstad (144). No explanation for these findings is yet apparent.

Inositol

Kirkwood & Phillips in 1946 (161) reported that growth of *Saccharomyces cerevisiae* was inhibited by γ-hexachlorocyclohexane and that this inhibition was reversed by inositol. More recently, Chargaff *et al.* (43) have reported that *meso*inositol, but not *d*-inositol or *d*-sorbitol, reverses the effects of γ-hexachlorocyclohexane and colchicine in the production of metaphase arrest and tumor formation in *Albium cepa* (onion) roots. Rosenberg (237) has reported that *meso*-inositol, but not *d*- or *l*-inositol, quercitol, rutin, fraxoside, rhamnetin, or hesperidin reverses the inhibitory effect of malonate on the growth of *Clostridium saccharobutyricum.*

The former two studies are in accord with the view that γ-hexachlorocyclohexane acts as an antimetabolite of inositol. However, its action is probably not this simple in view of the findings of von Vloten *et al.* (322) that inositol and γ-hexachlorocyclohexane do not correspond in their spacial configuration. Doisy & Bocklage (54) have reported that the toxicity of γ-hexachlorocyclohexane for rats is not reduced by the administration of

inositol. Although Lane & Williams (166) reported that the activity of a purified preparation of α-amylase, reported by Williams *et al.* (335) to contain inositol, could be inhibited by γ-hexachlorocyclohexane and restored to activity with inositol, Fischer & Bernfeld (75) found that crystalline α-amylase from pork pancrease or human saliva does not contain inositol, that inositol does not influence the stability of these enzymes in solution, and that enzymes are not inactivated by γ-hexachlorocyclohexane.

Gavin & McHenry in 1941 (88) reported that administration of a special liver fraction to rats on a fat-free diet induced a fatty liver characterized as "choline resistant-inositol susceptible." MacFarland & McHenry (180) now have shown that this type of fatty liver can be induced by the use of the additional B vitamins, folic acid, biotin, and choline in place of liver extract. These data apparently explain the earlier paper from the same laboratory postulating the existence in certain liver extracts of a separate factor concerned in the formation of fatty livers of the type studied.

Canepa, Grossman & Ivy (37) have produced additional data to indicate that the lipotropic activity of Dragstedt's lipocaic or Chargaff's antifatty-liver factor could not be accounted for by their contents of inositol or other known lipotropic agents [but cf. (332)].

Strepogenin

Stokes *et al.* (288) showed that the behavior of *L. casei* toward additions of trypsinized casein (strepogenin) varied markedly with the manner in which the culture of the test organism was carried. Strains which showed essentially no response to strepogenin were readily obtained. Other strains were obtained which (*a*) were stimulated greatly by addition of either asparagine or trypsinized casein to the medium, or (*b*) were inhibited by addition of asparagine, but in which this inhibition was counteracted by strepogenin. Antagonistic effects between asparagine and trypsinized casein (strepogenin) also were reported by Kodicek & Mistry (164). With very short periods of incubation, however, these investigators showed a growth stimulation by asparagine that was enhanced by strepogenin.

Rickes *et al.* (230) investigated the effect of modifications in composition of a semisynthetic medium, such as is commonly used in the assay of strepogenin, on the rate of growth of *L. casei*. The use of greatly increased amounts of asparagine, glutamic acid, and serine in combination and the addition of a reducing agent (ascorbic acid) gave a basal medium in which the rate of growth of *L. casei* was not increased by additions of trypsinized casein or of corn steep liquor. These data were interpreted as additional evidence for the probable involvement of these three amino acids as precursors of strepogenin. Asparagine was considered to function primarily as an amide donor, since Stokes *et al.* (288) have shown that trypsinized casein refluxed at a pH of 1 yields a product which shows strepogenin activity for *L. casei* only in the presence of asparagine.

Continued investigations of a possible growth-stimulating effect of strepogenin in animal rations have given negative results. Maddy & Elvehjem

(182) found that a ration containing 16 amino acids as the primary source of nitrogen gave essentially as good growth in mice as that obtained with intact casein, and the addition of intact proteins that are rich in strepogenin failed to improve the rate of growth obtained on the amino acid diet. Finally, diets containing a mixture of acid-hydrolyzed casein and intact casein gave no better growth than that obtained with hydrolyzed casein as the primary source of nitrogen.

Ramasarma *et al.* (225) similarly found that the growth rate of rats fed 20 per cent of acid-hydrolyzed casein supplemented with tryptophane and cystine was not significantly less than that of rats receiving intact casein. Brand & Bosshardt (19) found that a mixture of L-amino acids duplicating the composition of β-lactoglobulin (a good source of strepogenin by microbiological assay) was not inferior to the intact protein in supporting growth of the mouse.

Protogen, Pyruvate Oxidation Factor, Acetate Formation Factor, Vitamin B_{13}

An investigation of the Factor II requirement of *Tetrahymena geleii* W [Stokstad *et al.* (292)] showed that this factor could be separated into two components, IIA and IIB. The activity of the latter component was duplicated by a mixture of pyridoxal and copper ions [Kidder & Dewey (155)]. Stokstad *et al.* (292) suggested "protogen" as a tentative name for Factor IIA. Chromatographic techniques demonstrated that protogen exists naturally in at least two forms. As it occurs in a liver-norite eluate, protogen is essentially stable to autoclaving with 1*N* acid or 2*N* alkali, and may be extracted from water at pH 3.0 by butanol but not by amyl alcohol or chloroform. Protogen concentrates are inactive in promoting growth of chicks under conditions where a response is obtained with "animal protein factor" concentrates, and do not support growth of *L. lactis* Dorner. Thus there is no evidence for a relationship between protogen and vitamin B_{12}.

O'Kane & Gunsalus (208, 209) demonstrated that when *S. faecalis* 10Cl is grown on a specified synthetic medium, the cells obtained are deficient in the ability to oxidize pyruvate, but not glucose. The rate of pyruvate oxidation is greatly increased by the addition to the system of small amounts of a heat stable factor present in yeast extract. A few properties of the factor are given in the preliminary report (208). Their tabular data (209) indicate that concentrates of the pyruvate oxidation factor were obtained that were at least 200 times as active as the yeast extract used as a starting material.

Guirard *et al.* in 1946 (104) described the existence of an unidentified water-soluble factor that duplicated the growth promoting action of acetate for *L. casei* and certain other lactobacilli, but which was much more active, in crude concentrates, than was acetate. Snell & Broquist found (275) that concentrates of protogen and of pyruvate oxidation factor displayed this same growth-promoting action for *L. casei*. Three millimicrograms of a protogen concentrate purified 10,000 times over a standard liver preparation, as determined by assay with *T. geleii*, replaced 400 μg. of sodium acetate in

promoting growth of *L. casei* on an acetate-free medium. It was concluded (275) that protogen, the pyruvate oxidation factor, and the acetate-replacing factor for *L. casei* probably are identical. They cite unpublished data which indicate that one of the factors required for rapid growth of *S. faecalis* R from small inocula in acetate-free media [Cooperman *et al.* (47), Colio & Babb (46)], probably is this same compound.

The properties of vitamin B_{13}, as described by Novak & Hauge (203), are quite similar to those that have been reported separately for protogen, the pyruvate oxidation factor, and the acetate formation factor(s). Conceivably vitamin B_{13}, belongs to this group of closely related principles.

Miscellaneous Growth Factors

Bacterial growth factors.—A widely distributed substance required for growth by *Hemophilus parainfluenzae* was isolated and identified as putrescine by Herbst & Snell (119, 120). Spermidine and spermine were also highly active; arginine and ornithine were inactive.

An unidentified factor first reported by Sauberlich & Baumann (242) as essential for growth of *Leuconostoc citrovorum*, and present in refined liver extracts, migrates toward the anode of an electrolytic cell under conditions where vitamin B_{12} migrates toward the cathode [Lyman & Prescott (179)]. Furthermore, the *L. citrovorum* factor is stable to alkali under conditions which destroy vitamin B_{12}, and can be separated from the latter and from thymidine by chromatography on paper [Broquist *et al.* (25)]. Direct trials of vitamin B_{12} confirmed its inactivity (25). Much evidence relates this unidentified factor to folic acid. Thus, either folic acid or thymidine produces a slow, submaximal growth response under the same assay conditions (25, 242), and a mixture of folic acid and thymidine shows greatly enhanced activity (25). Ingestion of folic acid by rats leads to increased excretion of *L. citrovorum* factor, and concentrates containing the latter factor are effective in overcoming inhibition of the organism by aminopterin under conditions where folic acid is relatively ineffective [Sauberlich (241)]. Such data indicated that the *L. citrovorum* factor might well prove to be a metabolically active form of folic acid. This suggestion is borne out by the finding of Bond *et al.* (14), who recently indicated the existence of a group of substances other than folic acid, which they term "folinic acids," which counteract the toxicity of methylfolic acid for *L. casei*, more effectively than folic acid itself. Concentrates of one of these substances purified 200,000 times over an enzymatic digest of hog liver proved highly effective in promoting growth of *L. citrovorum* [Bardos *et al.* (7)]. Mild acid hydrolysis inactivated the substance for *L. citrovorum*, and liberated a compound indistinguishable from folic acid by microbiological tests.

Williams, Hoff-Jørgensen & Snell (339) describe an assay method and procedures for partial purification of a growth factor required by several strains of *Lactobacillus bulgaricus* and related lactic acid bacteria. The growth factor is not identical with vitamin B_{12}, various desoxyribosides, protogen, or the *L. citrovorum* factor. Ergostanyl acetate promoted slow and submaxi-

mal growth in the absence of the growth factor, but was less than 0.003 as active on the weight basis as crude concentrates of the factor. The substance is readily destroyed by acids and alkalis.

The protozoan organism, *Trichomonas vaginalis* requires an unidentified growth factor which could not be identified with any of the known vitamins or amino acids, or with strepogenin [Sprince, Gilmore & Lowy (283)].

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FAT-SOLUBLE VITAMINS[1]

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Vitamin A

Standardisation.—When β-carotene was adopted in 1934 by the 2nd Conference on Vitamin Standardisation as the international standard for vitamin A, only impure preparations of preformed vitamin A were available. Natural vitamin A, however, has now been obtainable in pure form for many years, and more recently equally pure specimens have been obtained by various synthetic procedures. Since many more biological tests are made on therapeutic preparations of vitamin A than on foodstuffs containing carotene, it has long seemed desirable to use preformed vitamin rather than carotene as the standard. This step had several advantages, especially in avoiding complications arising from possible variations in the efficiency of conversion of carotene to vitamin A. Experiences in using an unstable cod-liver oil such as the U.S.P. standard, however, suggested that great caution was necessary if similar trouble was to be avoided with pure Vitamin A.

Careful trials have now fortunately settled all doubts, and the Sub-Committee on Fat-Soluble Vitamins, appointed by the World Health Organisation (1), has fixed the unit at 0.344 μg. of a standard preparation of vitamin A acetate, equivalent to 0.3 μg. of vitamin A alcohol. Pure all *trans* vitamin A acetate, $C_{22}H_{32}O_2$, is defined as having M.P. =57.8–59.0°C. and $E^{1\%}_{1cm.}$ at 325 mμ. in isopropanol = 1,525, corresponding to 1,750 for the free alcohol. Ellenberger, Guerrant & Chilcote (2) have confirmed that identical preparations of the acetate can be produced either from natural sources or by synthesis in different laboratories, and that the vitamin, either in the crystalline form or diluted in oil, retains its full activity for prolonged periods in the absence of oxygen. The old standard of pure β-carotene has been retained for estimations of provitamins, the unit of 0.6 μg. being virtually identical in biological activity with the new vitamin A unit.

A new quantitative method for the estimation of vitamin A by the protection which it affords against degeneration in the central nervous system, as indicated by the Marchi technique, has been devised by Coetzee (3). It appears to give surprisingly accurate results.

Synthesis and Derivatives.—Isler *et al.* (4) have synthesised several esters and ethers of vitamin A, and have found them to be identical with specimens prepared from natural vitamin A. Good yields of vitamin A have been obtained by Schwarzkopf *et al.* (5) by the reduction of esters of vitamin A acid by lithium aluminium hydride. Van Dorp and his colleagues (6) have given

[1] This review covers the period from approximately January, 1949 to October, 1949.

details of their synthesis of vitamin A, and also of a *cis*-isomer of vitamin A aldehyde (7). Euler & Karrer (8) have found that axerophthene, the hydrocarbon corresponding to vitamin A, has about one fifth the biological activity of β-carotene.

An interesting derivative of vitamin A, probably the epoxide of retinene, has been isolated from the products of the oxidation of the vitamin with permanganate by Meunier & Ferrando (9). It has an absorption band at 345 mμ. and 4 per cent of the biological activity of vitamin A. Sharman(10) has confirmed that vitamin A acid is biologically active, although after dosing it disappears rapidly from the body without any evidence of conversion into vitamin A. Barua & Morton (11) have separated kitol esters from extracts of whale liver by a chromatographic method.

Carotene in vegetables, moulds, and oily media.—Mitchell, Schrenk & King (12) have found that carotene concentrates are usually unstable when carried upon finely ground solid materials; losses were less on soya bean and cottonseed meals than on casein, sorghum ground grain, sorghum bran, starch, or glucose. Lactic acid, but not lecithin or tocopherol, increased the stability of carotene on cottonseed meal. Methods of estimating carotene in vegetable sources have been examined in detail by several investigators (13, 14, 15). Thompson (16) has estimated carotene in numerous varieties and strains of alfalfa collected from plants of various stages of development, and has claimed that carotene concentrations, even on a dry weight basis, are 5 to 10 per cent higher in the early morning than later in the day. Haxo (17) reports that *Neurospora crassa* contains β-, δ-, and γ-carotenes, in addition to other carotenoids.

Holman (18) has investigated the coupled oxidation of β-carotene dissolved in ethyl linoleate at 37°C. The carotene was destroyed much more rapidly than by autoxidation in solvents such as butanol, but the spectral changes were qualitatively similar. The oxidation of the linoleate, as measured by the development of absorption bands at 268 and 233 mμ., commenced after the destruction of most of the carotene, and took the same course as in the absence of carotene. Using much the same methods, however, Chevallier, Burg & Manuel (19) found that carotene dissolved in triolein, containing 1 per cent of linoleic acid, acted as pro-oxidant in light but as an anti-oxidant in the dark.

Carotene in animals.—Goodwin & Srisukh (20) have found both β-carotene and astaxanthin in the integuments of locusts, but only β-carotene in the fatty tissues, blood, and eggs. No preformed vitamin A was detected in any of the tissues. In further work Goodwin (21) found that astaxanthin made up 70 per cent of the total pigments in the early hopper stage, but only 30 per cent in mature insects. Newly laid eggs contained 100 μg. per gm. wet weight of β-carotene, but during the later stages of pre-embryonic development β-carotene disappeared and astaxanthin was formed. The carapaces of lobsters and prawns (22) contained free astaxanthin, and their hypodermes larger amounts of esterified astaxanthin. Carotene was absent from these tissues, but was detected in small amounts in the hepatopancreas. Beatty (23) has found that the cavernicolous amphipod Crustacea of the

genus *Niphargus* do not contain carotenoid pigments even when they are available in their food, and ascribes their lack of pigmentation to the absence of light. Morton & Rosen (24) have examined the distribution of carotenoids and vitamin A in the tissues of tadpoles and frogs; vitamin A was stored mainly in the liver but the largest amounts of carotenoids were found in the ovaries. Grangaud & Massonet (25) again report that shrimp oil contains a factor, other than carotene or vitamin A, which cures xerophthalmia in rats deficient in vitamin A but is less effective in restoring their growth.

The most surprising development in this field, however, has been made in our knowledge of the source from which certain species of whales obtain their large reserves of vitamin A. Ten years ago Wagner (26) reported the isolation of crystalline β-carotene from the krill which form the food of Blue and Fin whales, and claimed that it was converted to vitamin A in the whale's intestines. Kon & Thompson (27), however, have failed to detect more than traces of carotene in the several species of krill which they have examined, but have observed substantial amounts of preformed vitamin A in addition to the characteristic astaxanthin pigments. This observation has important implications on the stage of evolution in animals at which vitamin A first appears. In view of Goodwin's observations on the variations of the relative concentrations of astaxanthin and β-carotene in locusts it might be unwise to discredit Wagner's claim to have isolated β-carotene from krill without fully exploring the effects of age and season.

Conversion of carotene to vitamin A.—In experiments with rats and pigs Thompson, Ganguly & Kon (28) have confirmed that carotene is converted to vitamin A in the small intestines. In the mesenteric lymph ducts the presence of the vitamin may be demonstrated by its fluorescence under ultraviolet irradiation soon after the oral administration of carotene. Cama & Goodwin (29) found that thyroxin and thiourea had no effect on the level of vitamin A in the plasma of rabbits. They could not confirm the alleged ability of iodinated casein or desiccated thyroid to convert carotene to vitamin A *in vitro* (30).

The absorption and mobilisation of vitamin A.—Eden & Sellers (31) have studied the absorption of vitamin A by bovines, sheep, and rats, and have confirmed the predominant importance of the lymphatic route. Goodwin & Wilson (32) have failed to substantiate the claim that the intravenous injection of epinephrine into rabbits causes the release of vitamin A from the liver into the blood, and have been equally unsuccessful with rats. Chapman, Gluck, Common & Maw (33) have observed large increases in the level of vitamin A in the blood plasma of immature pullets after injections of oestradiol diproprionate.

Vitamin A in animals.—Estimations of carotene and vitamin A in the rations of farm animals, and in their blood plasma, tissues, colostrum, and milk, have been made in extensive investigations on bovines (34 to 40), sheep (41, 42, 43), pigs (41, 44, 45), and poultry (46 to 49).

Sherman & Trupp (50) have continued their long-term experiments with rats to find out their optimum level of intake of vitamin A. Albino rats were

given a diet of five-sixths of ground whole wheat and one-sixth dried whole milk, supplemented with enough vitamin A in the form of cod-liver oil to bring the contents of the diet to 12 or 24 I.U. per gm. The length of life at both levels was 10 to 12 per cent longer than in previous trials in which 3 and 6 I.U. per gm. had been given, with a proportionally greater increase in the segment of the life cycle between maturity and senility. Since the response to 24 I.U. per gm. was slightly inferior to that with 12 I.U., it is assumed that the upper limit of the optimal range lies somewhere between these levels. Guerrant (51) found that daily doses of 44 I.U. were necessary for optimal growth in rats, which appears to correspond to the lower levels given by Sherman & Trupp, but this worked only observed ill effects with doses of 17,600 I.U. daily.

The vitamin A contents of seals' livers have been extensively investigated by Rodahl & Davies (52). The livers of mature hooded seals, *Cystophora cristata*, contained up to 24,000 I.U. per gm., with a mean of 3,000 I.U., while those of Greenland seals, *Phoca groenlandica*, contained up to 15,000 I.U., with a mean of 3,400 I.U.

Vitamin A and vision.—Substantial progress has been made in our understanding of the rôle of vitamin A in scotopic vision. Wald & Hubbard (53) have found that vitamin A may be obtained on bleaching cell-free extracts of ox or frog retinas only in the presence of an enzyme system. This is necessary for the reduction of retinene, identified by Morton as vitamin A aldehyde, into vitamin A alcohol. In *Rana pipiens* this system could be fractionated anatomically. Thus the outer segments of the rods, removed from the underlying retinal tissues by scraping, were only able to convert their retinene to vitamin A after the addition of an extract, either of crushed retinas or of skeletal muscle. The activating agent present in the extracts was fairly stable to heat, and could be replaced by reduced cozymase, or cozymase supplied with the addition of fructose diphosphate. The system can now be set up with synthetic retinene prepared by the mild oxidation of vitamin A, purified reduced cozymase, and an apoenzyme in the form of a clear saline extract of homogenised retinas (54). A similar system may also be set up with retinene$_2$ prepared by the oxidation of vitamin A_2, reduced cozymase and an apoenzyme preparation made from the retinas of fresh water fishes. Retinene$_2$, however, may be reduced equally well with apoenzyme from frog retinas, while retinene$_1$ may be reduced with apoenzyme from fresh water fishes. The same retinene reductase is therefore effective in both the rhodopsin and porphyropsin cycles.

Cozymase can be protected by nicotinamide or α-tocopheryl phosphate. The retinene reductase system, as most effectively assembled *in vitro*, therefore involves intricate interactions between three vitamins. As Wald points out, the main process presents the novel phenomenon of one vitamin regenerating another, in that the DPN-H_2 which reduces the retinenes to vitamin A, contains nicotinamide, a member of the vitamin B_2 group, as its central component. While so engaged DPN-H_2 is protected from cleavage by the presence of free nicotinamide. The latter is aided in this action by

vitamin E phosphate, which simultaneously protects the vitamin A formed in the main reaction from oxidative destruction.

The rôle of enzymes in promoting the reverse reaction in which vitamin A is converted to retinene has been studied by Bliss (55). Since the vitamin is an alcohol which can be dehydrogenated to a typical aldehyde it seemed probable that the well known reversible DPN-specific alcohol dehydrogenase might be involved. Crystalline vitamin A, dispersed with a detergent, was therefore mixed with an extract of rabbit liver, with coenzyme I as hydrogen acceptor, and with bisulphite or cyanide to act as aldehyde trapping reagents. When the retinene was subsequently released from its addition compound by dilution, or by treatment with alkali, yields of up to 40 per cent were obtained by extraction with light petroleum.

Artificial products spectroscopically similar to indicator yellow, a product of the bleaching of rhodopsin in which retinene is still loosely bound to protein, have been obtained by Ball, Collins, Dalvi & Morton (56) by coupling retinene in alkaline solution with amino compounds and proteins. They have also obtained substances resembling rhodopsin by treating retinene in acid solution with aniline and similar compounds. Ball & Morton (57) have found that solutions of retinene or vitamin A in concentrated sulphuric, phosphoric or hydrochloric acids show sharp absorption bands characteristic of unstable ionised molecules, and simulating the photopic modulators of Granit. They suggest, therefore, that the vitamin A-retinene system may be concerned in photopic as well as scotopic vision.

Vitamin A in the human. A full report has now been published of the important investigation on vitamin A deficiency carried out in Britain during the war under the auspices of the Medical Research Council (58). Out of 27 volunteers 16 were restricted to a diet from which all foodstuffs containing more than traces of carotene and vitamin A were omitted, while 7 received the same diet but with supplements of carotene or of vitamin A. In all the volunteers, except those given carotene, carotenoid pigments disappeared almost completely from the blood plasma during the first few weeks of the experiment. The decline in the plasma vitamin A, which ranged from 65 to about 120 I.U. per 100 ml. at the start of the experiment, was much less rapid, and in one volunteer the original level was maintained for almost two years. For the other subjects the average of 88 I.U., found soon after the commencement of the experiment, only fell to 74 I.U. in nine months, and in seven subjects who were kept on the experiment for 14 months was still as much as 61 I.U. The declines in both carotenoids and vitamin A were prevented or reduced in volunteers who were given daily doses of 2,500 to 6,700 I.U. of carotene in various forms. The fall in vitamin A, but not in carotenoids, was prevented by doses of 2,500 I.U. of a distilled concentrate of vitamin A esters.

In parallel tests of dark adaptation there was some evidence of seasonal variation in all the subjects, the final rod thresholds reflecting the variations in the monthly averages of the daily minimum temperatures. More serious deteriorations in dark adaptation, not related to season, were found in three

of the undosed volunteers, who all had plasma vitamin A levels of 40 I.U. or less. Only in these three cases were satisfactory therapeutic tests possible. Preformed vitamin A, in daily doses of 1,300 I.U. over a long period, both corrected dark adaptation and restored the level of vitamin A in the plasma to about its original level. With a daily dose of 1,250 I.U. of carotene in oily solution, the levels of vitamin A and carotenoids in the plasma were slightly increased, but doses of 2,500 I.U. were necessary to improve dark adaptation. The efficiency of absorption of carotene from carrots and green vegetables was studied in several volunteers by finding the differences between the amounts eaten and those excreted in the faeces. When allowances are made for wastage during absorption, and for an adequate safety margin, the daily requirement of a male adult may be assessed as 2,500 I.U. of preformed vitamin A, or in the form of carotene as 4,000 I.U. in oily solution, 7,500 I.U. as green vegetables or 12,000 I.U. as boiled sliced carrots.

Apart from the three instances of defective dark adaptation the volunteers showed no symptoms which could be definitely ascribed to vitamin A deficiency, although during the experiment two of them were found to be suffering from tuberculosis. This freedom from symptoms seems less surprising when it is recalled that the liver reserves of vitamin A in cases of accidental death in Britain had been found before the war to be sufficient to meet the body's requirement for at least several months. Further data, included in the present report, gave no indication of decreased reserves as the results of wartime dietary changes.

Chieffi & Kirk (59), in studies on elderly hospital inmates, found higher incidences of hyperkeratosis of the skin and of local conjunctival thickening in a group of subjects with 1 to 15 μg. of vitamin A per 100 ml. of blood plasma than in another group with 25 to 60 μg. Veghelyi & Lancos (60) have observed keratomalacia and a low plasma vitamin A in a young child infected with *Giardia lamblia;* his uninfected brother remained normal although receiving the same diet. Houet & Weekers (61) have reported signs of vitamin A deficiency secondary to liver abnormalities in infants.

Vitamin A deficiency in animals.—Eveloth, Bolin & Goldsby (62) have kept yearling ewes on a diet deficient in vitamin A. Some died suddenly without symptoms, others, which had been deficient for about a year, developed incoordination and lordoses. The production of wool and meat, however, was little affected. Newly weaned lambs succumbed in three to four months when given the same diet. Fletcher & Rigdon (63) observed neurological manifestations, associated with bone changes, within seven to eight days after the restriction of newly hatched ducklings to a deficient diet. Anderson (64) noticed a high incidence of diaphragmatic hernia in young rats whose mothers received inadequate amounts of vitamin A.

Hypervitaminosis A.—Further proof that the toxicity of polar bear liver is due to its high content of vitamin A has been advanced by Rodahl (65, 66) who has found that the toxicity is associated with the vitamin A fraction, and not with the residual tissues after the extraction of the vitamin. The liver of the Arctic fox, which the Eskimos believe to be poisonous, was found to approach bear liver in its vitamin A content, whereas the edible livers of

the walrus and snow hare contained much smaller amounts. In work on various experimental animals Rodahl (67) has also obtained more detailed evidence of the similarity between the lesions sustained in hypervitaminosis A and in scurvy. In dogs given a large excess of vitamin A, Maddock, Wolbach & Maddock (68) observed loss of appetite, skeletal changes, hyperaesthesia, and exophthalmus. Irving (69) compared the effects of avitaminosis and hypervitaminosis A upon the incisor teeth and incisal alveolar bone of rats, and concluded that the action of vitamin A is chiefly on the osteoblasts and odontoblasts, whose activity is greatly increased or reduced according to whether the vitamin is lacking or in excess. Studer & Frey (70) observed that the skin on the backs and abdomens of rats given large excess of vitamin A was considerably thickened, while in contrast the skin of deficient animals was atrophic and abnormally thin.

Vitamin A and detoxication.—The influence of vitamin A on the detoxication of sodium benzoate by rats has been studied by Meunier *et al.* (71). Animals receiving a diet deficient in vitamin A survived and grew well when given supplements of 2.5 μg. of carotene, but grew slowly and eventually died with the same supplements when 2 per cent of sodium benzoate was added to their diet. Good growth and survival could be secured even if benzoate were given, however, either by raising the carotene supplement to 20 μg. daily or by counteracting the benzoate with corresponding doses of glycine.

VITAMIN D

Standardisation and biological assay.—The standard for vitamin D, adopted in 1931 by the Health Organisation of the League of Nations, consisted of a solution of irradiated ergosterol containing a certain proportion of vitamin D_2. Crystalline vitamin D_3, made by the irradiation of 7-dehydrocholesterol, has now been recognized as identical with the natural vitamin, and has the advantage of being equally active in mammals and in birds. It is now available in adequate quantities, and according to a recent decision of the Sub-Committee on Fat-soluble Vitamins of the World Health Organisation (1) will henceforth replace the old vitamin D_2 standard. The International Unit will be equivalent to the activity of 0.025 μg. of the standard preparation of vitamin D_3, and the old solution of vitamin D_2 will be preserved until the stability of vitamin D_3 has been verified.

In assaying vitamin D for poultry, Hubbell, Bliss & Nolan (72) have studied the effect of the fat solvent used to prepare the bones as a preliminary to ashing. Slightly different percentages of ash were found according to whether carbon tetrachloride or a combination of alcohol with ether was used for the extraction. In extensive tests, however, it was found that equally precise results were obtained with either solvent, provided it was used consistently. Snyder, Eisner & Steenbock (73) have suggested a method for the estimation of vitamin D based on its effect on the degree of radioactivity observed in the paws of rats after the intraperitoneal injection of radioactive phosphorus.

Chemistry and biochemistry of antirachitic substances.—Scott, Glover &

Morton (74) have detected a sterol with an absorption spectrum typical of the provitamins D in the lining of the small intestine of the guinea pig, rat and ox. In guinea pigs this sterol made up 3.6 per cent of the non-saponifiable fraction, as compared with 0.5 per cent in the skin. It persisted in the gut wall of guinea pigs when they were fasted for 24 hr. or were kept on a diet low in sterols for two weeks, and was temporarily increased when cholesterol, spectroscopically free from provitamins, was administered. It is concluded, therefore, that 7-dehydrocholesterol is formed in the gut wall by the dehydrogenation of cholesterol.

Velluz and his colleagues (75, 76) have continued their studies on precalciferol, which is obtained as a main product of the irradiation of ergosterol in the cold. This interesting substance has now been isolated as a dinitrobenzoate which differs from that of calciferol in its crystalline form, optical rotation, and extinction coefficient at 265 mμ. Precalciferol, either free or as the dinitrobenzoate, is unstable to heat when in solution and is changed into calciferol at the temperatures which are usually reached in concentrating solutions of irradiated ergosterol. Calciferol, on the other hand, is partly converted to precalciferol when heated in solution, but it is still the predominating isomer when the equilibrium point is reached. An analogous precalciferol$_3$ has been obtained from dehydrocholesterol. Somewhat similar phenomena have also been reported by Raoul, Chopin, Meunier & Le Boulch (77). When calciferol was dissolved in an ionising reagent, consisting of symmetrical dichloroethane with the addition of glycerol dichlorohydrin, there was a displacement of the absorption spectrum towards longer wave lengths until the typical spectrum of tachysterol appeared. If at this point alkali was added, and the ionising solvent was replaced by light petroleum, only slight heating was necessary to regenerate calciferol. If the ionising action was continued too long, however, the tachysterol became stable, and calciferol could not be produced again except by irradiation.

Yoder & Thomas (78) have obtained substances with some degree of antirachitic activity by refluxing cholesterol in acetic acid solution with chlorosulphonic acid. Antirachitic products were also obtained from certain unsaturated and oxidised derivatives of cholesterol, and from the sterol of maize germ oil, but not from the sterol of sperm oil, lanosterol, or isocholesterol.

Chemical and physical estimation of ergosterol and calciferol.—Ettinger & Sobel (79) have devised a method for the estimation of ergosterol in small specimens of yeast by means of the greenish yellow colour which it produces with activated glycerol dichlorohydrin. The colorimetric estimation of calciferol in oily solutions by means of the antimony trichloride reagent has been studied by Nielsen (80), while Pirlot (81) has explored the practicability of estimating calciferol by infrared spectrophotometry. The possible value of microbiological methods for the estimation of D vitamins is under investigation by Kodicek (82).

Mode of action.—As the result of experiments with chicks, some involving the use of radioactive calcium, Migicovsky & Emslie (83) have concluded that the primary effect of vitamin D is to promote the retention of calcium in

the bone. Lillie & Bird (84) have found that some breeds of chicks are more susceptible than others to the abnormal blackening of the feathers which sometimes occurs as a result of deficiency of vitamin D. Bourne (85) has drawn attention to evidence that the preen glands of birds and the sebaceous glands of animals may be important in secreting provitamins, which are exposed to sunlight and subsequently consumed during the normal procedures of toilet.

Vitamin E

International Conference.—A most successful conference (171) organised by Dr. Karl E. Mason under the auspices of the New York Academy of Sciences, and timed to take place almost exactly 10 years after the first international conference in prewar London, was not only an outstanding scientific event but also an inspiration to all those privileged to attend. Representatives from Britain, Canada, Germany, Holland, Italy, and South America contributed towards the 68 communications which were included in the two-day programme.

Chemistry.—Since the quinone formed by the oxidation of α-tocopherol is not reconverted into the vitamin on reduction, Michaelis & Wollman (86) have sought some other oxidation product capable of functioning in atypical oxidation-reduction process. On theoretical grounds it seemed possible that a semiquinone radical might be formed by the oxidation of a single hydroxyl group without opening the side chain. To obtain practical proof of the existence of this radical α-tocopherol was dissolved in a mixture of alcohol, ether, and pentane, which was frozen to a vitreous mass, by liquid air. By ultraviolet irradiation an orange-red colour was produced, which showed a characteristic absorption spectrum. The colour persisted after the cessation of irradiation, but faded on melting the frozen solvent. Boyer, Rabinovitz & Liebe (87) extended a previous investigation in which it was found that after tocopherol had been oxidised with ferric chloride, it could be recovered intact by prompt reduction with ascorbic acid, but not if the reduction was unduly delayed. The reversibly oxidized product was isolated by chromatographic methods, and was found to differ from α-tocopherol in containing one more oxygen atom, and in being about 30 times less active in biological tests. Its spectroscopic behaviour and its ability to liberate iodine from sodium iodide, suggest that it is an epoxide. Probably the production of the semiquinone of Michaelis & Wollman is the first step in its formation.

Vitamin E and enzyme systems.—Ames & Risley (88) have suggested, as a working hypothesis, that vitamin E may act both specifically through some enzyme system and nonspecifically as a general physiological antioxidant. They discuss three types of investigations; first, the effect of vitamin E deficiency on enzyme systems, second, the related problem of the effect of tocopherol when added to enzyme systems *in vivo* and *in vitro*, and third, the separate question of the effect of the artificial water-soluble phosphate of tocopherol on enzyme systems. Since the phosphate is a strong detergent, and can also immobilize calcium, the interpretation of experiments in the third category always presents difficulties.

According to Ames & Risley (88) the phosphates of α-, γ-, and δ-tocopherol all inhibit the succinic oxidase system, while calcium partially counteracts their inhibitory powers. A high concentration of free tocopherol was also found to inhibit the system for several hours. Roderuck, Basinski & Barber (89) observed a decrease in transaminase activity in dystrophic muscles from deficient guinea pigs and rabbits, and consider that the greatly increased oxygen uptake of the diseased tissues may be due to the excessive oxidation of metabolic products such as glutamic acid, which usually undergo transamination.

In experiments on hyaluronidase by Miller & Dessert (90) phosphates of the various forms of tocopherol had the same relative order in inhibiting the enzyme as in biological tests with rats: $\alpha > \gamma > \delta$. Zierler *et al.* (91) found that large doses of α-tocopheryl phosphate in rats produced mascular weakness, depression of basal oxygen consumption, and depression of endogenous respiration in the skeletal muscles but not in the liver. The contractibility of isolated nerve muscle preparations was considerably affected by injections of α-tocopheryl phosphate.

Phosphoryation systems, however, seem to have been the most popular field of research. Thus Govier & Gibbons (92) found that α-tocopheryl phosphate decreased the formation of phosphocreatine by homogenised normal guinea pig hearts; the reduced rate of phosphorylation found in the hearts of animals deficient in vitamin E was affected differently according to the amount of phosphate added and the concentration of potassium ions. No reduction in adenosinetriphosphatase (ATP) or in alkaline phosphatase was found by Carey & Dziewiatkowski (93) in the muscles of deficient rabbits, but the acid phosphatase activity was about doubled; ATP and acid phosphatase in both normal and diseased muscles were inhibited by α-tocopheryl phosphate unless calcium was added.

Aldman *et al.* (94) report that α-tocopheryl phosphate by itself slightly inhibits the alkaline phosphatase of juvenile rabbits' kidneys, but that it also completely removes the inhibition by oestradiol-3:17-diphosphate; it slightly decreased inhibition by progesterone but had no effect on inhibition by oestrone-3-phosphate or phloridzin. In the same animal, according to Heinrich & Mattill (95) deficiency of vitamin E causes an increased rate of synthesis of creatine in the liver, with a decrease in the rate of its phorylation in the muscles.

The genital organs in vitamin E deficiency.—Atkinson, Kaunitz & Slanetz (96) have made the interesting observation that rats deficient in vitamin E do not develop the typical brown pigmentation of the uterus if their ovaries have been removed. The uteri became brown if the ovariectomized animals were injected with oestradiol, but progesterone had no effect except partially to neutralize the action of oestrogen. Elftman, Kaunitz & Slanetz (97) consider that the brown pigment is probably produced by the peroxidation and polymerisation of unsaturated fat. The increase of collagenous fibres in the uteri of aging rats, according to Lopes de Faria (98) is not influenced by the presence or absence of tocopherol. Pierangeli, Radice & Herraiz (99) have

confirmed that the testicles of deficient rats contain a fluorescent lipopigment similar to that seen in the uterus.

Kaunitz and his colleagues (100) have also commented on the increased requirements of rats for tocopherol during the menopause, and on the restoration of vaginal oestrus in old rats by tocopherol (101). The lengthened oestrous cycles in aging rats could be corrected by giving liberal doses of the vitamin. The failure to conceive in old rats which are deficient in vitamin E appears to be due to disturbances in uterine implantation of the ovum rather than to failure in ovulation, or in the transport of ova through the oviducts (102).

Other effects of deficiency.—Luttrell & Mason (103) have confirmed that rats kept on a diet deficient in vitamin E develop paresis which is associated with muscular and nervous lesions. When the lard component of their diet was oxidised by aeration it became toxic, but although it caused the rats which ate it to become debilitated, their muscular and nervous lesions were less pronounced than in animals given fresh lard. Malamud, Nelson & Evans (104) also confirmed the reality of the nervous lesions in chronic vitamin E deficiency. Filer *et al.* (105) found slight but consistent electrocardiographic and pneumonocardiographic abnormalities in *Macaca rhesus* monkeys submitted to prolonged deficiency of vitamin E, but no other abnormalities were observed. Bragdon & Levine (106) have detected foci of acute myocarditis at necropsy in rabbits kept on a diet deficient in vitamin E.

Protection by vitamin E in conditions of metabolic stress.—The role of vitamin E as a protective agent presents one of the most intricate and fascinating problems in the whole field of biochemistry. Repeated experiments have indicated that tocopherol may counteract the toxic effects of certain highly unsaturated fatty acids, such as those of cod-liver oil. It also helps animals to resist the effects of protein deficiency and affords protection against liver injuries of nutritional origin. As a consequence of these interrelationships we are faced with a complicated overlapping of at least three major fields of research on (*a*) the injurious effects of unsaturated fats, (*b*) nutritional liver injuries, as influenced, *inter alia* by sulphur compounds, and (*c*) the rôle of protein, choline, and other lipotrophic agents in preventing the accumulation of fat in the liver. The present review can only hope to make brief allusions to work on various separate aspects of the protective action of tocopherol, without attempting to present a complete and balanced picture.

Dam and his colleagues (107 to 111) have continued studies in which they have shown that tocopherol prevents the action of cod-liver oil, and other highly unsaturated fats, in causing peroxidation and brown pigmentation in the adipose tissues of rats and chicks. A histological method for the demonstration of peroxides in the tissues has been devised (107).

The effect of vitamin E in protein deficiency has been studied by several workers. Himsworth & Lindan (112) have reported that tocopherol prevents massive necrosis of the liver in rats given a diet with yeast as the main source of protein, and hence defective in amino acids containing sulphur.

With a somewhat similar diet given to adult rats for a prolonged period, Moore (113) found that tocopherol prevented liver necrosis, anaemia, and persistent loss of weight, but not heavy infiltration of the liver with fat. By giving their rats a diet containing soya protein, Matet, Matet & Fridenson (114) have also produced liver necrosis, which could be prevented either by tocopherol or by methionine. Schwarz (115) considers that there are three kinds of liver injury, all preventible by tocopherol, which may beaused by diets containing (*a*) protein purified by alkali, (*b*) excessive amounts of cod-liver oil, or (*c*) yeast as the main source of protein. Working with mice, Menschik & Szczesniak (116) have found by histological methods that tocopherol increases the amount of neutral fat deposited in the liver, but prevents the formation of globules of lipo-protein.

The protective action of tocopherol against poisoning by carbon tetrachloride has been examined by Hove (117). The symptoms of massive lung haemorrhage, liver necrosis, blanching of the incisors, and reduced growth, which followed in his rats from a diet deficient in vitamin E, were accelerated in development by the administration of the poison, but in spite of such abuse the injuries could be prevented by tocopherol. Single, sublethal injections of carbon tetrachloride into rats caused a creatinuria and hypocreatininuria, as found in animals deficient in vitamin E. György & Rose (118) observed that rats given a basal diet deficient in vitamin E were protected by tocopherol against the early death with haemoglobinuria which otherwise followed injections of alloxan. The erythrocytes of deficient animals were not haemolysed *in vitro* by alloxan, but were readily haemolysed by dialuric acid unless tocopherol was added.

Pindberg (119) has found that rats given a diet deficient in vitamin E, and containing succinylsulphathiazole, may be protected against dental abnormalities either by tocopherol or by the consumption of faeces from rats given a normal diet; he assumes that under certain circumstances vitamin E may be synthesised by the intestinal flora.

Farm animals.—Numerous workers have studied the importance of vitamin E in the nutrition of cattle (120 to 126), sheep (120, 127, 128), goats (128) pigs, (120, 128, 129, 130), and horses (126), and have followed the variations in the blood, colostrum, and milk under different nutritional conditions and during different phases of the reproductive cycle. Gullickson (123) has found that when cattle are deprived of vitamin E their reproductive capacity is little affected, but that sudden death through heart failure often occurs. Whiting, Willman & Loosli (127) have reported that ewes given a diet of red kidney beans with alfalfa or clover hay often produce lambs with stiff joints, which may be cured by dosing with tocopherol. Since the total tocopherol of the defective diet was no lower than those of other diets which did not cause stiff joints, it would appear either that the beans contain mainly the less active forms of tocopherol, or that their tocopherols are inefficiently utilised. Adamstone, Krider & James (129) found that the reproductive performance of sows kept on a diet deficient in vitamin E was greatly lowered, apparently as a result of the death of the embryos. In those piglets which could be reared muscular incoordination, caused by disintegra-

tion and necrosis of the muscle fibres, was frequently observed. According to Jungherr (131) deficiency of vitamin A must not be overlooked as a poslible cause of field encephalomalacia in chicks.

Human blood and tissues.—Quaife & Dju (132) have evolved a method for the estimation of vitamin E in tissues. The tissues were usually frozen in liquid air, ground while solid, and then extracted with ethanol; if rich in fat, however, they were homogenised with the solvent in a Waring blendor. Vitamin E was estimated by the ferric chloride method in the extract after concentration, molecular distillation and the destruction of carotenoids by dehydrogenation. The body of a man, killed by accident, contained a total of 2.31 gm. of tocopherol, while that of a woman contained 6.56 gm. The most important stores of tocopherol were located in the adipose tissues, but the vitamin was present in all the tissues examined. The greatest concentrations per unit of fat were found in the testes and uterus. Quaife, Scrimshaw & Lowry (133) have devised a micromethod, dispensing with hydrogenation and distillation, which allows the estimation of total tocopherol in a few drops of blood obtainable by finger tip puncture. After parturition in women who were not dosed with vitamin E, Neuweiler (134) found somewhat lower levels of the vitamin in the venous cord blood than in the maternal blood, but much lower levels in the arterial cord blood. The same indications of the absorption of vitamin E by the foetus were observed in mothers who had been dosed with the vitamin, but the levels were higher in all specimens.

Engel (135) has reviewed earlier work in which, with his colleagues, he observed levels of about 0.8 mg. per 100 ml. of the blood serum of persons given 15 mg. of tocopheryl acetate daily. Levels of more than 1.2 mg. were seldom observed, and this level was not exceeded in a subject dosed with 120 mg. daily. The experiments indicated that when 15 mg. daily of tocopherol was given, in addition to the 15 mg. estimated to be present in the diet, the maximum blood level was reached in a few days. The requirement for a maximum level in the blood, therefore, appears to be about 30 mg. per day. Low levels of tocopherol were found in new born babies and in sprue patients, but not in cases with neurological or gynaecological diseases.

Darby *et al.* (136) have also estimated tocopherols in the blood of large numbers of healthy and diseased subjects. Low values have been found in diseases characterised by impaired absorption of fat and high values in metabolic disturbances involving hypercholesterolaemia. The levels in heart disesaes were usually about normal, but high values were often found in hypertensive cases. During pregnancy the level of tocopherol in the plasma steadily increased, but no consistent variations were observed in relation to the menstrual cycle. In a similar investigation Popper *et al.* (137) found no correlation between the contents of the plasma in vitamin E and in vitamin A. Scrimshaw, Greer & Goodland (138) have made a special study of tocopherol levels in pregnancy; the levels found in women aborting between the 17th and 24th weeks of pregnancy were significantly lower than those found for the same period in women who did not abort, but at other stages of pregnancy no difference was found in the aborting and normal groups.

Clinical trials.—While experimental evidence of the importance of vitamin E in the nutrition of animals has continued to accumulate, and has proved that the vitamin affects many tissues and many biochemical systems, our knowledge as to its value in human nutrition remains empirical and highly controversial. Although Harris (141) has emphasised the wide variations in potency to be found between different therapeutic preparations of vitamin E, it seems unlikely that the sole cause for divergent clinical experiences can be found in this direction.

Only limited success can be claimed in the treatment of muscular diseases which, by analogy with lesions found in experimental animals, might be expected to respond to treatment with the vitamin. Thus Bicknell (139) observed no improvement, as judged by creatine and creatinine excretions, in patients with muscular dystrophy or amyotrophic lateral sclerosis who were treated with vitamin E and inositol combined with desiccated preparations of stomach or duodenum, and with or without folic acid. Milhorat *et al.* (140) however, claim to have isolated tocopherylquinone from the oxidition products of a specimen of hogs' mucin which had been found effective in reducing creatinuria in a patient with progressive muscular dystrophy. The possibility that the metabolic defect in this disease may be traced to an inability to convert tocopherol in tocopherylhydroquinone has therefore to be considered.

Heart disease.—Shute's claim that tocopherol is valuable in the treatment of heart diseases, particularly as manifested by angina pectoris, have been challenged by Travell *et al.* (142), Eisen & Gross (143), Ravin & Katz (144) and Baer, Heine & Gelfond (145). Although Lemley *et al.* (146) found slightly reduced levels of tocopherol in the plasma of cardiac patients equally low values were found in miscellaneous patients without heart disease. Shute, however, has remained confident that his claims are well justified, and has supported them in an account of the treatment of coronary heart disease (147), and in a review of the physiological and biochemical basis underlying the use of the vitamin in cardio-vascular diseases (148).

Venous obstructions and ulcerations.—Shute (149) has also continued to advocate the use of tocopherol in the management of acute and subacute vascular obstructions, and in the treatment of burns. Venous obstructions were usually resolved after massive doses had been given for periods extending from a few days up to several months, and dosing could usually be discontinued within a few weeks of the healing of the lesion. Patients with stasis ulcers or dermatitis resulting from chronic venous insufficiency caused by varicose veins or chronic thrombophlebitis were treated with tocopherol by Stritzler (150). This treatment seemed to be helpful in most of the cases with dermatitis and some with ulceration, but it was usually necessary to supplement the oral doses with the injection of aqueous emulsions homogenised with Tween. Pennock (151), however, considered that the progress of ulcerations due to chronic venous insufficiency in patients treated orally with tocopherol was much as might have been expected without its administration.

Arterial diseases.—In Shute's experience (149) tocopherol was valuable

in the treatment of thromboangiitis obliterans, although a heavy rate of dosing had to be continued permanently. Boyd, Hall Ratcliffe & James (152) considered that the results which they obtained with tocopherol in the treatment of obliterative vascular diseases at least justified further trials, while later Hall Ratcliffe (153) found in controlled experiments that the walking abilities of patients with intermittent claudication were significantly increased by tocopherol. Pennock (151), however, could discern no benefit in advanced cases of thromboangiitis through treatment with tocopherol, while Lippman (154) was equally unimpressed with its value in arteriosclerotic peripheral vascular disease.

Collagenous abnormalities.—Claims that tocopherol is often beneficial in Dupuytren's contracture of the fingers have been made by Steinberg (155) and by Russell Thomson (156). Some success appears also to have been achieved in the treatment of Peyronie's disease of the penis by Steinberg (155) and by Scardino & Scott (157). According to Steinberg (155) tocopherol may also be valuable in the treatment of rheumatic fever. Ant & Di Cyan (158) suggest that it counteracts muscular disorders of rheumatic origin.

Miscellaneous diseases—Vogelsang (159) has reported that vitamin E is beneficial in diabetes, both in reducing the insulin requirement and in combating cardiovascular complications. This claim has been supported by Butturini (160) but has been reputed by Guest (161) and by Densley *et al.* (162). According to controlled tests by Dowd (163) tocopherol is effective in the management of Sydenham's chorea, and also beneficial in multiple sclerosis (164). Scardino & Hudson (165) claim good results in urethral stricture. McLaren (166) recommends tocopherol for the control of menopausal flushing and sweating when the administration of oestrogens is contraindicated.

Vitamin K

Davis *et al.* (167) have reported that 2-methyl-1,4-naphthoquinone is unstable when exposed in dilute aqueous solutions either to a mercury arc lamp screened to emit radiations at 366 mμ. or to direct sunlight. Its destruction may be prevented, however, by small concentrations of chloride ions. Bromide ions are also protective, but not fluoride or sulphate ions. Schopfer & Grob (168) found that the activity of urease preparations was inhibited by naphthoquinone derivatives, such as 1,4-naphthoquinone and 2-chloro-1,4-naphthoquinone, which oppose the action of vitamin K, and which have high activity in suppressing the growth of microorganisms. The inhibition of the enzyme by these anti-vitamins could be prevented by the simultaneous addition of either sulphur compounds such as sodium sulphide, cystine or glutathione, or of naphthoquinone derivatives with vitamin K activity. Axelrod, Cooper & Brodie (169) have developed a method for the estimation of dicumarol in plasma or urine by acidification and extraction by heptane, followed by the extraction of the heptane with dilute alkali and measurement of the absorption at 315 mμ. Meunier (170) has examined the activities of diphthiocol and phenylindanedione as anti-K vitamins in rabbits, and has found them to be much less potent than dicoumarol.

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NUTRITION[1]

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Introduction

More than 1,500 references were collected for the past year, although all papers that might be mentioned elsewhere in this volume were excluded. The number of references for the review on Nutrition has averaged 162 annually during the past seven years. If a third of the permitted printed pages be devoted to references, some 250 may be included, which is less than 17 per cent of those collected. Reluctantly, sections on the following have had to be omitted: clinical allegations regarding vitamin E, nutrition and infection, therapeutic uses of massive doses of calciferol, canine hysteria, obesity, dietary factors and atherosclerosis, diet in hypertension, effect of nutrition on pregnancy and foetal malformations, international aspects of nutrition, clinical aspects of vitamin C and of rutin, anti-vitamins including dicoumarol, nutritional oedema, and experimental calorie deficiency. On all these subjects important work has been done; on the last, the promised book summarising the classical work of Keys has not yet appeared which gives a slight justification for omission.

Throughout, emphasis will be upon the application to man of biochemical work on nutrition. If the maelstrom of papers published during the year is moving in any particular direction, a tendency may be discerned toward a more critical and rational use of fundamental work on lower animals, applied cautiously to man when malnutriture is found in patients or in communities and when it is purposely induced experimentally. The pendulum continues to swing from vitamins to calories and protein and beyond to amino acids; and there is a suggestion that in a short time it will swing back from amino acids to essential peptides and so to vitamins. Particular attention will be paid in this review to dietary protein and amino acids in relation to disorders of the liver and blood. Though nutritional deficiencies are usually multiple, it is becoming realised that pure deficiencies occur and can be caused by anti-vitamins, as in Chastek paralysis in silver foxes or bracken disease in cattle. A more critical approach to the assessment of nutriture in man has shown the folly of a too hasty application of half-truths for public health purposes. There has also been far too much haste to publish the experimental work with man and lower animals upon which the science of nutrition must be founded.

Dietary Surveys

The usual purpose of a dietary survey is two-fold: first, to assess the food eaten by an individual or group of individuals; secondly to estimate the nutritional composition of this food.

[1] This review covers a twelve month period beginning October 1948.

Methods.—Consumption of food may be computed by different methods, and the discrepancies between them have been further investigated. In a valuable study, Bransby and colleagues (37) applied to a group of 49 children in an institution four methods of individual dietary survey: weighing, questioning, homely measures, and chemical analysis. In most instances the food eaten was overestimated by use of homely measures and considerably underestimated by the questioning technique. Compared with chemical analysis, the other three methods gave overestimates for calories, fat, carbohydrate and all nutrients except iron, which was underestimated; protein was underestimated by weighing and questioning but the estimate by homely measures agreed with chemical analysis. The same authors (38) made a second study of three-day diets of 33 adults living at home in four towns. The average group values for calories, protein, fat, carbohydrate, and calcium agreed reasonably well with those found by chemical analysis; but for individual diets the methods of analysis and computation gave results so divergent as to throw doubt on the usefulness of the individual results obtained by computation.

Another comparison (131a) of the nutritional value of diets, as computed from tables and analysed, showed that despite careful treatment of a kind that cannot be applied in nutritional surveys of populations, whereby allowances for losses in cooking and like factors might be made, unreliable results were obtained: for instance, one diet was found by analysis to contain 2.0 mg. of ascorbic acid despite a computed value of 120.0 mg. [one might well wonder why the figure after the decimal is included]. Similarly, Toscani (273) obtained large differences between calculated and analysed figures for 12 individual diets; and Collins (61) found that during rigid rationing of food in Vienna in 1946, 24-hour dietary histories gave an estimate of food consumption that was smaller than the ration and much smaller than was reflected in the nutriture of the population. Loss of food by plate-waste in the Army (10) and cooking losses (13, 85) have been measured.

It is abundantly clear that the incidence of nutritional deficiencies in a population cannot be assessed from dietary data alone; these can do no more than indicate the broad trends of consumption of food or nutrients. Dietary surveys are expensive, and those contemplating their use would do well to ponder over the valuable information regarding techniques and their limitations that is now available.

Food composition.—The most important publication under this heading is the *Food Composition Tables for International Use* prepared from 151 reports by Chatfield (92). These tables give average values for calories, protein, and fat per 100 gm. of retail weight; and also water, protein, fat, carbohydrate (by difference and fibre), ash, and calories per 100 gm. of edible portion, together with refuse in retail weight. Calories were calculated on the principles enunciated previously by the F.A.O. (*Food and Agriculture Organisation*). Vitamins and minerals are not yet included. No doubt in many countries locally prepared tables will continue to be used since they

should, of course, be more accurate; but the present tables are a valuable asset to the majority of countries which have none of their own.

The composition of Chinese foods has been determined by Harris and co-workers (116), and the tables of composition of Australian foods have been revised (208a). A new edition of the Netherlands Food Tables has appeared (74); this gives data obtained in Dutch laboratories for the nutritional composition of food as purchased. An interesting study (223) compares the amino acid content of a simple Dutch diet with breads of 75 per cent and 100 per cent extraction; and amino acid analyses for different food stuffs are beginning to appear more frequently (144, 191). The essential amino acids in milk have been estimated (164), and a very comprehensive review of milk has been published (154); various nutritional aspects of milk were discussed at the XII International Dairy Congress in Stockholm. The use of soya for infant feeding has received additional attention (75), and further papers have appeared on the following foodstuffs: food yeast (39, 77, 78, 99); cereals (59, 137, 294); rice (90, 206, 236); whale (17); potato (57)—particular mention must be made of the scholarly review of the potato by Salaman (235). Mack (184) has continued her detailed study of the relative value of meat and of legumes in the feeding of growing children in two orphanages; certain clinical signs seem to be diminished by increased consumption of meat, and growth and skeletal mineralization are improved. Problems of synthetic fats for human consumption have been discussed (160, 198, 270); F.A.O. (91) has reviewed their potential contribution to world food requirements, and has emphasised that, although expensive, their manufacture in Germany should be encouraged and further experimental work should be vigorously developed. The chemist becomes increasingly important in the fight to feed the world.

Results of dietary surveys.—Nine brief papers by Mašek, Kruta and colleagues record food consumption surveys in Czechoslovakia, particularly of adolescents whose nutriture is also assessed (162). Assessments have been made of the food eaten by hospital patients (21, 131), pregnant women (48, 76), children (11, 94), students (152), the aged (218), and soldiers (240, 265). Phipard & Stiebeling (216) have reviewed the adequacy of diets in the United States: the consumption of dairy products, citrus fruit, and vegetables (except potatoes) has risen greatly since 1909, whereas the consumption of potatoes and grain-products has fallen; the computed per capita consumption is now as high in all and much higher in many of the nutrients listed in the National Research Council allowances, which are generous allowances set sufficiently high to cover the requirements of substantially all the population; Maynard (193), however, believes calcium may be deficient in many U.S. dietaries. A fifth report of *Food Consumption Levels in the United Kingdom* (93) carries this series to the end of June 1949; approximately the same amounts of nutrients seem to "move into civilian consumption," and when these are compared with those of the U.S. (216) it seems that the U.K. wins only in calcium. Kraut (156) has compared the

consumption of calories, protein, and fat by the German people in 1909 to 1913, 1935, 1940, and 1947 with the official Bizonal plan of December 1947: the levels of calories and protein suggested by the plan, which appear to be higher than the N.R.C. allowances, are considered to be slightly too low; the 69 gm. per capita daily of fat is criticised. The adequacy of the Berlin ration has also been discussed (121).

A dietary survey has been conducted in Dublin as the first part of the National Nutrition Survey (133). It is stated that "in 1944, the question arose of finding out what was the level of nutrition of the population of Ireland"; in fact, the question had often been asked before. The Department of Local Government and Public Health conducted surveys between April and August 1946, from which it was concluded that broadly the intake of nutrients,with the exception of calcium, was satisfactory at all levels of income, of food expenditure and of family size; it would seem that no allowance was made for calcium in water, but Dublin water is not hard. The most comprehensive surveys of diet are those published by the Section of Nutrition of the Institut National d'Hygiène in France under Trémolières (275). These, in general, show increasing and satisfactory levels of consumption; in Paris a return to white bread (80 per cent extraction) increased the consumption of bread with a corresponding fall in that of meat and fat, and the part played by bread in the French diet has been discussed at length by Trémolières (274).

Nevertheless, it must be emphasised that dietary studies such as these, valuable though they are for certain purposes, tell us nothing about the incidence of malnutriture in the population.

Hunger and appetite.—In a stimulating review, Lepkovsky (176) has summarised work on the physiological basis of voluntary food intake. He analyses the changes in the internal medium of the body which are accompanied by changes in the acceptability of food, and the elaborate mechanism whereby an increase or decrease of food intake is effected. Nutrients may be divided into three classes according to the response to them of an animal deficient in them: there is an immediate response to some such as sodium or phosphorus, suggesting that the taste mechanism is involved; there is a delayed response to others, such as thiamine, which is presumed to be associated with a chemical reaction in the taste mechanism to the deficient nutrient; there is little or no response to others such as amino acids and certain vitamins. Harte and others (117) have also found that growing rats have no protein-hunger mechanism; growing rats showed a close relation between calorie intake and surface area, regardless of the protein quality of the ration. Other aspects of food selection and hunger have been studied (9, 16, 81, 138, 139, 195, 233, 250). The importance of bulk in the diet has been emphasised again (35); in rats, bulk-formers prolong life and prevent diverticulosis (52, 53).

Mention should here be made of the final paper by Tisdall and his colleagues (272) on the food parcels packed and shipped by the Canadian Red Cross Society for prisoners-of-war in Europe and the Far East—some 16½

million parcels at a cost of $47,529,000. Interviews with 6,551 men were obtained shortly after their return to Great Britain and completed questionnaires were analysed in Canada: biscuits, butter, meat, milk, and chocolate were especially appreciated in that order.[2]

Nutritional Requirements and Allowances

General.—There has been considerable activity in the field of nutritional or dietary standards. A new edition of the *Recommended Dietary Allowances* of the National Research Council (202) is an event of importance, and a new dietary standard has also been introduced for Canada (50a; 215). The standards adopted during the war by the Oxford Nutrition Survey have also appeared in print (252); these were used for providing a nutritional unit for calculating diets, and therefore standards for animal protein, fat, carbohydrate, phosphorus, carotenoids, and vitamin A were separately included. Dietary requirements or allowances from various sources are often compared but are often not comparable. Those of the N.R.C. "are intended to serve as a guide for planning an adequate diet for every normal person of the population and not merely for the average member of the group categories"; it is stressed that they are not the minimal requirements of average individuals, "but levels enough higher to cover substantially all individual variations in the requirements of normal people." The Canadian standards seem to represent probable physiological requirements for maintenance of full health of average individuals of different age, sex, activity, and weight, and "to indicate a 'nutritional floor' beneath which the maintenance of health in individuals cannot be assumed"; these standards, like those of the N.R.C., are stated to be based on amounts actually ingested, but it is unlikely that this is true of calories. The nutritional requirements of the Oxford Nutrition Survey may be defined as follows: the requirement of a nutrient is the estimated amount, expressed as a daily average over a whole year, that must be ingested by the average person within the specified ranges of age, sex, activity, and body-size, in a temperate climate, to keep that person in normal nutriture and therefore also to permit adequate growth; in the case of aliments and of energy, the figures refer to the amounts actually assimilated (that is, physiologically available). It seems therefore that the Canadian and Oxford standards refer to the average person, whereas those of the N.R.C. might be regarded as being higher by at least twice the standard deviation from the average. Despite this there is substantial agreement, which is surprising. The problem of biological variation and normality is raised (145, 161, 253). We know little about the causes of variation in requirements of persons of the same age, sex, activity, and body-

[2] The death of F. F. Tisdall deprives nutritional science of a clinician and biochemist whose enthusiastic work was so largely responsible for this magnificent organisation. At the same time the deaths of W. J. Dann, whose biochemical researches showed refreshingly constructive criticism, and of W. A. Perlzweig (also at Duke University) who contributed so much to the biochemistry of pellagra, are regretfully recorded.

weight, but they include such factors as the composition of the diet, the somatotype, and the mechanical efficiency of the person. If the N.R.C. allowances are to cover the requirements of substantially all normal persons, whatever the variation in those factors, one would expect them to be considerably higher than the other two standards. In general they are the highest, the Canadian standards being generally the lowest. The most marked discrepancy between the three comes in ascorbic acid. For instance, in the age group 0 through 1 year, the N.R.C. allows 32 mg., Oxford 10 mg., and Canada 30 mg.; for a moderately active man the respective figures are 75, 30, and 30; for a lactating woman they are 150, 90, and 30. It is difficult to believe with the Canadians that an infant and a lactating woman require the same daily amount of ascorbic acid, but comment on the Canadian figures should be suspended until the full statement concerning them becomes available. They are based upon sex and weight for children; a useful paper on the Canadian and other dietary standards has been published by Young (301), and a brief note on the biological basis of such standards by Crampton (62). Scheunert (238, 239) has reviewed the troublesome question of human requirements of ascorbic acid and has described an extensive experiment on German workers supplemented with different amounts of ascorbic acid (20 to 300 mg. daily), thiamine, vitamin A, and quinine; the number of illnesses in 242 days per 100 workers is given: it is highest for those receiving 50 mg. of ascorbic acid daily and significantly lowest for the two groups receiving most ascorbic acid, namely 100 mg. and 300 mg. Scheunert concludes that the daily requirement is very high, perhaps 125 mg. Papers have appeared on the nutritional requirements of children (43, 263), and on the feeding of soldiers (63, 147, 175, 188, 247, 281) and lower animals (45, 46, 47, 51, 127, 241, 249). The detailed study of human requirements of vitamin A (194) is mentioned elsewhere in this volume.

Calories.—Rather over half the calorie allowances listed by the N.R.C. have been reduced slightly (taking account of changes in body-weight at different ages); the N.R.C. has also reduced slightly its allowances of B-vitamins, and has increased adult calcium allowances. The calorie requirement of adult man has been admirably reviewed by Keys (146). As a result of nutritional experience during the war and in postwar Europe, which largely confirmed previous beliefs about calorie requirements (80), there is a salutary return to the importance of calories (42). Heat production in relation to metabolic size has been studied by Galvão (97, 98), and a useful and simple method of calculation for indirect calorimetry allowing for protein metabolism has been introduced by Weir (290); Van Veen (283) has discussed calorie conversion factors with special reference to south east Asia. The activities of children at play (267) and of housewives at work (79) have been scrutinised.

Protein and amino acids.—Active interest in dietary protein has continued, and in a historical review McLester (186) calls this the protein era; he believes that the 140 gm. of protein consumed daily by the U. S. soldier contributed to the winning of the war. An important series of papers on the

protein requirements of heavy workers has appeared from the Dortmund Institute (157, 159, 173). Jonxis (66) found that a supplement of protein added to the ordinary rations of children aged 2 to 6 years in the summer of 1947 improved their growth and haemoglobin percentage, whereas an equicaloric supplement of carbohydrate was unfavourable. The great importance of adequate dietary protein for surgical patients continues to be stressed (192, 225, 291). Using the tube-feeding technique of Lathe (169), Peters's work on the dietary conditions affecting urinary loss of nitrogen in burned rats has been continued: both cold and burns increase the nitrogen excretion in rats fed a controlled diet but the two effects are not additive and methionine does not diminish the excess nitrogen excreted (170); feeding sucrose produces a large reduction in excretion of nitrogen, which leads the authors to suggest that extra calories in this form may be advisable for patients to prevent protein wastage (171). Two clinical cases have been presented (181) which are stated to indicate that DL-methionine may be worthy of trial in stubborn unhealed surface wounds in protein-depleted humans, one a boy aged 10 years with third-degree burns and the other a man of 77 years with chronic ulcers of the ankles; the same paper shows that whereas methionine does not speed the healing of surface wounds of normal rats, it restores towards normal the prolonged healing-time in protein-depleted rats, despite continued depletion. Mitchell (200) has analysed data obtained by him in 1907 to 1908, when 23 university students, aged from 18 to 31 years, took part in a metabolism experiment for up to 220 days; the balances of nitrogen and of phosphorus were consistently positive and were positively correlated with each other and with the surface area of the men; apart from excretion of nitrogen through the skin, about 1 gm. of nitrogen may be considered as being retained for growth of skin, hair, and nails. In exfoliative dermatitis there may be considerable losses of protein, and a case has been reported illustrating the importance of protein therapy (142). Whedon and his colleagues, who earlier showed (72) that immobilisation of healthy individuals in a plaster cast could produce negative nitrogen balance, have found that the metabolic effects of immobilisation can be considerably decreased by using a Sanders oscillating bed (295). A new functional syndrome of obscure aetiology and pathogenesis has been described (129) in which hypoproteinaemia, oedema, and peripheral vascular changes occur.

Ramasarma and colleagues (221) observed the growth of weanling rats receiving rations containing mixtures of purified amino acids, acid-hydrolysed casein, or intact casein. Improvement of growth resulted from the addition of "nonessential" amino acids to adequate amounts of the "essential" ones. Under the conditions of the experiment, no evidence was obtained that young rats require a source of strepogenin for growth.

The effect of varying the quality and quantity of dietary protein on the pattern of urinary excretion of amino acids in different species has been studied. In mice and rats, the proteins that promoted the most rapid growth were metabolised with least loss of microbiologically available amino acids in the urine (237). In human subjects, histidine and threonine were excreted

in large quantities which appeared to bear a positive relation to their intakes, whereas the excretion of glutamic acid, aspartic acid, tryptophane, and methionine, which were present in fairly large quantities, seemed to be independent of the amount ingested (260). Kirsner *et al.* (151) also observed a direct correlation between dietary content and urinary excretion of histidine and threonine in man; urinary excretion of other amino cids did not parallel their varying intakes. In addition they found that, with the exception of methionine, there was no direct parallelism between dietary amino acids and their concentration in plasma. Eckhardt & Davidson (82) observed that the urinary excretion of the free and combined forms of the individual amino acids continued throughout an 8-day period during which a healthy subject received adequate calories but no protein. Increases in the quantity of protein ingested resulted only in small increases in the quantity of amino acids excreted, showing thereby that a large portion of the amino acids in the urine of subjects eating ad libitum may be of endogenous origin. Baldwin & Berg (14) observed that in the human subject, D-tryptophane was definitely inferior to L-tryptophane in maintaining adequate nitrogen balance and that acetylation of the D-isomer did not enhance its value; this finding is in contrast with the observation of Albanese *et al.* (4) in male infants that D-tryptophane on acetylation becomes as effective as L-tryptophane. Rose (232), however, has shown that neither D-tryptophane nor its acetyl derivative exerts any detectable influence upon nitrogen balance in man. Almquist (5) found that the amount of total methionine needed to support a maximum rate of growth of chicks was proportional to the level of dietary protein. Thus he found that the methionine requirement of the chick for maximum growth on a 30 per cent protein diet was approximately 0.75 per cent, and on a 20 per cent protein diet requirement was 0.5 per cent. Brown (44) reported that both methionine and thiouracil increased the total nitrogen content of the liver in rats; methionine increased the nitrogen per gram without affecting the size of the organ, whereas thiouracil increased the size of the organ without affecting the nitrogen per gram of tissue. Chow *et al.* (58) reported that the difference in the ability of hydrolysates of casein and of lactalbumin to regenerate plasma protein in protein-depleted dogs was not due to the difference in the amount of absorbed nitrogen, but perhaps to the existence of an unidentified nutritional factor. It should be noted that the excretion of amino acids in the urine represents a loss of calories. It has often been observed that pair-fed animals deficient in or receiving B-complex vitamins grow at different rates, although they ingest and absorb identical amounts of aliments and their activities are comparable. If oxidisable substances such as amino acids are lost in the urine, this result would obtain particularly in the case of nutrients such as pyridoxine that are concerned in amino acid metabolism.

The dietary requirements of amino acids in man have been studied by Rose during the past seven years. With commendable caution and restraint all too rarely found in biochemistry, he has deliberately postponed publication of his results until they were sufficiently numerous and definite to war-

rant positive conclusions. His paper (232) is undoubtedly the most important published in the field of nutrition during 1949. Strictly tentative values for the recommended daily intake (being twice the minimum daily requirement) are given for the eight L-amino acids required by healthy male students: tryptophane, phenylalanine, lysine, threonine, valine, methionine, leucine and isoleucine. Abundant nitrogen for the synthesis of nonessentials was supplied by glycine or urea, about 10 gm. of nitrogen daily usually being supplied in the food. As Rose points out, it should now be possible to evaluate proteins in terms of their ability to meet human needs, and successful intravenous alimentation for certain patients becomes much more feasible, as he first suggested almost 15 years ago.

Parenteral feeding.—Parenteral feeding is necessary in persons with gastrointestinal abnormalities who are unable to ingest or digest or absorb sufficient quantities of food. In recent years, attempts have been made to develop a complete intravenous diet containing adequate amounts of water, minerals, vitamins, carbohydrate, protein, and fat. Such efforts have been handicapped by difficulty in obtaining an emulsion of fat suitable for intravenous use. In this connection the observations of Meng & Early (196) are of particular interest since they have maintained four healthy adult dogs for as long as four weeks on a diet given exclusively by vein. The diet furnished proportional amounts of carbohydrate, fat (in a fine emulsion), protein, minerals, and vitamins. The dogs remained healthy and lively, and gained weight. Several physiological tests during the experimental period and final histological examination of the organs at autopsy revealed no significant changes attributable to this procedure. Another interesting feature was that one dog, given the same treatment except fat, lost 14 per cent of its initial weight, became emaciated, and developed typical skin lesions suggestive of fatty-acid deficiency, showing thereby that the injected fat was used. Meng & Early therefore conclude that the emulsion can be used clinically with considerable confidence. That intravenously-injected fat is utilized was shown by Stare and his colleagues (102) by the conclusive experiment of injecting trilaurin containing C^{14} carboxyl-labelled lauric acid into fasted rats and measuring the radioactivity of the respiratory carbon dioxide collected subsequently; 70 per cent of the injected C^{14} was recovered in $4\frac{1}{2}$ hr. as expired carbon dioxide. By injecting the 18-carbon palmitic acid containing C^{14} in the sixth carbon atom into two rats, Lerner *et al.* (177) arrived at the same conclusion. In further work, Stare and his colleagues (189) observed no toxic reactions in six human subjects as a result of intravenous injection of a fat emulsion (coconut oil) within 25 days of its preparation; after 25 days, however, pyrogens developed on the emulsion. These results are certainly encouraging, but more experimental proof of the safety of fat emulsions administered intravenously is needed before clinical applications become widespread.

Protein hydrolysates and amino acid mixtures by intravenous route are being used with increasing frequency, although there is no definite evidence that they are superior to intact protein by mouth. Eckhardt & Davidson

(82a) found that intravenous injection of hydrolyzed serum albumin supplemented with L-tryptophane was adequate for a man as a source of protein. Williams and her colleagues (299) reported favourably on the intravenous use of a protein digest in the treatment of very sick infants. Cohen & Ma (60) recommend "hyperalimentation" with protein hydrolysates in tuberculous patients with advanced lesions or in whom operative measures are contemplated, but the results reported by the authors with this regimen seem to be inconclusive. Vollmer (287) obtained encouraging results with hydrolysates given orally in chronic empyema and gastric ulcer. Zweig *et al.* (302) studied the effect of intravenous protein hydrolysates on gastric secretion and motility. Gastric acidity was increased in 55 per cent of 106 tests, but gastric motility remained unaffected. Neither vagotomy nor thoracic sympathectomy inhibited the gastric response. Reference is made below to the use of intravenous amino acid mixtures and protein hydrolysates in the treatment of cirrhosis of the liver.

Assessment of Nutriture

Reviews of methods.—During the past few years too many nutritional surveys have been conducted and too little thought has been given to the methods used and the interpretation of the results of those methods. A nutritional survey that does not in fact assess nutriture is worse than useless—worse because erroneous conclusions are drawn and nutritional science is derided. During the year two comprehensive reviews of the assessment of nutriture have appeared (203, 253). The National Research Council has published a very valuable Bulletin on the techniques and interpretation of nutritional surveys, prepared by a Committee under the chairmanship of C. G. King (203); amongst other subjects, the Bulletin treats of dietary surveys, chemical tests, and clinical diagnosis each of which receives a quarter of the space; the remaining quarter is mainly applications to public health. My review (253) does not cover applications, but devotes over a third to a theoretical discussion of nutriture, a third to clinical methods of assessment, a seventh to functional methods such as dark-adaptation, a tenth to chemical methods, and the remaining tenth to dietary and somatometric methods.

Both reviews agree that dietary surveys cannot in general be used alone to assess nutriture; the N.R.C. contrasts dietary surveys and nutritional surveys. Regarding clinical methods, both reviews agree that there is no single pathognomonic clinical sign of nutritional deficiency with the exception that I have stated—and continue to believe—that perifollicular petechiae are characteristic of deficiency of ascorbic acid. A useful check-sheet for clinical signs in nutritional surveys is given by the N.R.C., but it is not arranged in a form suitable for punching on Hollerith cards; that used by the Oxford Nutrition Survey is so arranged that the results can be sorted on the needle-system or punched on Hollerith cards without further coding, and it has been reproduced (252, pp. 268–69). The differences between them need not be detailed except for one very important point: the N.R.C.

makes no provision for recording either the extent of examination of the skin or the absence of teeth, and yet the incidences of skin signs in different surveys cannot be compared unless the same areas are examined, and the incidence of gingivitis is unknown unless the number of edentulous persons is recorded, since they do not develop gingivitis in nutritional deficiency. It may be mentioned that the very important work of Kraut & Muller on the relation between coal production and caloric intake is mentioned by the N.R.C. which assesses the results "despite the patriotic incentive to produce maximally during a war"; the Russian prisoners working in the Ruhr were sometimes given cigarettes as an incentive but certainly did not love the Vaterland.

The main difference between the two reviews regarding chemical tests is the emphasis given to urinary estimation: both regard blood estimations as of more importance than urinary, but the N.R.C. is more optimistic about the use of the latter in rapid surveys; however they note that the only tests of urinary excretion that can be used in surveys are those requiring brief periods of collection, which eliminates excretion after a test dose; and since in surveys it is often difficult to obtain specimens during fasting, the use of urine seems very limited. The N.R.C. believes urinary "saturation" tests "will determine the degree of saturation or depletion of the tissues with a given vitamin," but if vitamin A be given this is not true; it is believed that "saturation suggests at least adequate nutrition," but—as is later stated—those who have recently had access to nutrients sufficient to raise tissue concentration without restoring function or reverting certain pathological changes may be nearly "saturated." In general the chemical section of the N.R.C. is an excellent compilation, and much of it has been reproduced elsewhere by Goldsmith (107); one might regret that the N.R.C.'s appendix of useful tests omits mention of B-complex vitamins in leucocytes and of ascorbic acid in blood (which is much more valuable than in plasma in nutritional surveys), and disagree with the frequent mention of estimating calcium in blood, since most workers prefer serum. There is no doubt that, with increasing realisation of the uselessness of dietary surveys for assessing nutriture and of the unspecificity and lateness of clinical signs, chemical analyses upon blood or a part of blood (such as leucocytes, which afford a biopsy of living cells) will occupy an increasingly important position. The uncertainty of the clinical evaluation has been admirably shown by Bean (19), who has also described requirements for a field test of rations (18). The technique of chemical estimations of vitamins has been reviewed in an article by Harris (115) and in books by Pittaluga (217) and Villela (284). Titration of serum with mercuric chloride (36), cutaneous injection of mercuric cyanide (168), and estimation of serum cholinesterase (182, 183) have been suggested for assessing aspects of nutriture; the last was introduced by McCance and promises to be a useful method of assessing caloric deficiency.

Kruse's "concept."—In an earlier review in this series (167), Lanford & Sherman devoted considerable space to Kruse's *A concept of the deficiency states*, published in 1942; this has been criticised in the review mentioned

above (253). A long article on *A concept of the etiological complex of deficiency states with especial consideration of conditions* has now been published by Kruse (161). In this Kruse developes "a new concept" of the aetiology of the deficiency process. In the opinion of the reviewer, what is correct in the new concept is already generally accepted and is here dressed in such verbosity as to make it almost unrecognisable; and what is new is found, when stripped of its pedantic camouflage, to be incorrect. In the article "Considered especially are the conditions: their nature, association, action, effects, and significance"; the conditions are factors that alter the supply of a tissue nutrient or the requirement of a tissue for it; yet "a condition is something that necessarily precedes a result, but does not produce it." A thrombus will alter the supply of tissue nutrients, will precede a result in the form of necrosis of tissue, and certainly should be regarded as having produced that result. A "tissue essential is a nutrient that a tissue must receive for its nutrition" and includes nutrients synthesised in the body (including endocrine secretions) and dietary essentials; the latter are nutrients "that must be present in the diet if nutrition is to be successful," yet β-carotene is stated to be a dietary essential although it certainly does not come within this definition. Once more we meet our old friends from Kruse's earlier concept: velocity, intensity, and duration. And again we find confusion. For instance, we are told that "the previous velocity of the force $\times$ acceleration = new velocity of the force"—which is obviously untrue since the dimensions are wrong: $v_1(dv_1/dt) \neq v_2$. These concepts should be studied carefully and judged on their merits.

Results of assessments of nutriture.—The results of a large number of nutritional surveys have been reported during the year, but considerations of space unfortunately prevent examination of them. Surveys of various merit and complexity have been conducted, or the incidence of supposed or real deficiencies reported, in the following regions: Africa (2, 40, 41, 207), Alaska (122, 214), Australia (11), Brazil (70), Canada (141, 286), Ceylon (33, 64), China (130), Czechoslovakia (162), Denmark (135), Egypt (1), Far East (251), Fiji (271), Germany (15, 22, 26, 27, 28, 84, 110, 172, 208), Great Britain (3, 126, 243), Greece (259), India (220), Mexico (8), Netherlands (204, 223, 224), Newfoundland (12, 107, 242), South America (210), Spain (285), and the United States (277, 278, 279).

Special mention may be allowed for the extensive report edited by Burger Drummond & Sandstead, on *Malnutrition and Starvation in Western Netherlands, September 1944–July 1945* (204), particularly since the two volumes seem to be difficult to obtain. Although, as stated in the foreword, "the report on the famine is not the main object of this publication," a wealth of information is included from Netherlands clinicians, biochemists and survey teams; it is intended that the clinical and biochemical data should be published in extenso in separate publications. From the biochemical aspect, the most important work is included in about 100 pages in the first volume and in an appendix of 300 pages. The account of the work of the Netherlands Pols Commissie is followed by that of the three nutrition survey teams

formed by the U. S. Army, the Royal Canadian Air Force, and the Oxford Nutrition Survey; these accounts should provide useful information about the conduct of rapid nutritional surveys. An excellent report by de Jongh of the clinical features of starvation is followed by the biochemical investigations on patients in the Test Ward in The Hague and in several Dutch laboratories: it is remarkable that the Netherlands biochemists achieved so much with so few facilities during the famine. The results of the team from the Oxford Nutrition Survey, which was joined by a large number of Netherlands biochemists, are next described: some 25,000 biochemical estimations of various types were made. The whole of the work shows the results of gross undernourishment from acute deficiency of aliments, the Calorie intake of adults at times falling to 600 daily; there was little evidence of vitamin deficiencies apart from some deficiency of vitamin A and of niacin. Famine oedema was common, particularly in the higher age-groups, but the serum proteins of these persons usually fell within the normal range.

Malnutriture and Haemopoiesis

Nutritional factors in haemopoiesis.—It is now known that a number of nutritional factors are concerned in haemopoiesis, and a lively discussion about these was an outstanding feature of the first International Biochemical Congress in Cambridge, England, in August 1949. Wintrobe, for instance, has during the past 14 years conducted studies in which about 1,100 young swine have been fed a purified diet of which the individual constituents could readily be altered. Of the various substances studied deficiencies of pyridoxine, pteroylglutamic acid, iron, and protein appear to influence erythropoiesis in swine most profoundly, and niacin and riboflavin to a lesser extent. Other factors have been shown to be effective in animals, but not all claims rest on a sure basis. For instance, it is concluded (228) that thiamine is essential for normal haemopoiesis in monkeys, but the claim is based on haematological studies on four animals which had a markedly reduced food intake early in the experiment, were not pair-fed, and showed only a small reduction in erythrocytes and haemoglobin. The sections that follow will be devoted entirely to clinical applications of biochemical work.

Vitamin B_{12} and pteroylglutamic acid.—The isolation from liver of crystalline anti-pernicious-anaemia factor, tentatively named vitamin B_{12}, simultaneously by Rickes and co-workers (226) and Smith (254) constitutes one of the greatest advances in this field. The chemical aspects of this are reviewed elsewhere in this volume. Using the material isolated by Rickes, West (292) obtained good haematological responses in three patients with pernicious anaemia in doses of the order of a few micrograms, and this observation was soon confirmed (32, 111, 140, 256, 293). The evidence so far published also shows that the neurological complications of pernicious anaemia are favourably influenced by vitamin B_{12} (24, 32, 112, 140, 258, 293). Vitamin B_{12} is also effective in controlling the lingual inflammation and atrophy of pernicious anaemia (246, 262). Thus it appears that, where pteroylglutamic acid (PGA) failed, vitamin B_{12} is capable of completely replacing

potent injectable liver extracts in dealing with the haematological, neurological, and lingual manifestations of pernicious anaemia. Dedichan & Laland (71), however, recently pointed out that whereas liver extracts act on granular white cells, this vitamin does not. Using the material isolated by Smith in the largest series of cases of pernicious anaemia yet published (53 cases), Ungley (280) found that a single dose of 10 mg. produces in 15 days, both in respect of reticulocytes and of increases of erythrocytes, as good a response as is usually obtained with liver extracts; larger doses gave even better results.

Among other macrocytic anaemias, Spies and his colleagues (258) obtained typical remissions in four cases of nutritional macrocytic anaemia of southern U.S.A. and eleven of West Indian sprue; Patel (212) reported good responses to treatment with Smith's crystalline factor in two cases of nutritional macrocytic anaemia occurring in Bombay; McPherson *et al.* (187) reported the successful use of B_{12} in two cases of megaloblastic anaemia of infancy. In contrast with these results, Bethell *et al.* (32) have reported a case of puerperal macrocytic anaemia that showed no response to vitamin B_{12} but did respond to subsequent administration of PGA. Day *et al.* (69) also failed to elicit improvement with vitamin B_{12} in one case of macrocytic anaemia of pregnancy, but obtained excellent clinical and haematological improvement from subsequent parenteral administration of PGA. In the experimental macrocytic anaemia produced in the rat by the formation of a cul-de-sac in the small intestine, Witts and his co-workers (50) found that PGA was more effective than vitamin B_{12} or purified liver extract. Thus in macrocytic anaemias other than the pernicious type, vitamin B_{12} does not appear to be uniformly effective, and more extensive trials of the vitamin in them are clearly required.

At the same time, the mode of action of the vitamin is being unravelled. Berk, Castle and co-workers (23) and Bethell *et al.* (32) have reported that large faecal losses of vitamin B_{12}, detected by microbiological assay, occur in untreated cases of pernicious anaemia. Callender *et al.* (49) found that a faecal extract possessed similar activity to vitamin B_{12} when injected into a patient with pernicious anaemia. Berk and co-workers (23) made the interesting observation, confirmed by Hall, Morgan & Campbell (113), that 5 μg. of B_{12} found to be inactive by mouth, can be made to yield reticulocyte responses when administered together with neutralised human gastric juice. These observations suggest that vitamin B_{12} may actually be Castle's extrinsic factor and that the patient with pernicious anaemia is unable to absorb this vitamin in the absence of the intrinsic factor. It is now known that vitamin B_{12} is active on oral administration in large doses (257).

The announcement of the isolation of the anti-pernicious-anaemia factor of the liver has naturally been followed by reports of others working on the growth-promoting fractions of liver. For several years there has been evidence that certain laboratory animals, namely the rat, chick, and dog, maintained on vegetable protein concentrates or highly extracted casein as

the source of protein, require additional growth factors which occur primarily in animal products. Recent evidence suggests that vitamin B_{12} and the "animal-protein factor" are closely related. Spies *et al.* (255) reported the beneficial effects of a concentrate of animal-protein factor on persons with pernicious anaemia, sprue, and nutritional macrocytic anaemia. Ott, Rickes & Wood (209) demonstrated that vitamin B_{12} had growth-promoting activity for chicks maintained on a soya-protein diet. Lillie, Denton & Bird (178) obtained similar chick growth responses to purified cow-manure factor, liver extract, and vitamin B_{12}. It is known that rats, ingesting desiccated thyroid gland, require in addition to all the known vitamins a growth-promoting substance which is present in liver: Betheil & Lardy (31) now present evidence to show that vitamin B_{12} acts as a growth factor for thyroid-fed rats. Hartman *et al.* (118) also reported a good growth response on administrat on of vitamin B_{12} to their "x-deficient" rats. On the microbiological side, Shive *et al.* (248) reported the isolation of a factor from hog liver that counteracted the toxic action of methyl-PGA upon the growth of *Leuconostoc mesenteroides* 8293. This substance was identified as thymidine, the desoxyriboside of thymine. Wright *et al.* (300) reported that thymidine could replace B_{12} as a growth factor for certain lactic acid bacteria—a subject that is reviewed in detail elsewhere in this volume. These observations point to a functional relationship of PGA, vitamin B_{12} and thymidine. Clinically, thymine has been shown to be haemopoietically active in human macrocytic anaemia (96). Hausmann (120) mentions beneficial therapeutic effects of thymidine administration to two patients with pernicious anaemia. Reisner & West (222), however, obtained only slight reticulocyte responses, but no effect on erythrocyte counts in three cases of pernicious anaemia treated with thymidine. Thus rapid advances are being made in this field and much of what is here written may become out-of-date at the time of publication.

Niacin.—No conclusive proof of any specific haemopoietic activity of niacin in man is yet available. In weanling pigs, Wintrobe (55) produced a normocytic anaemia by feeding them on a low-protein diet deficient in niacin. The anaemia responded to the administration of a high-protein diet or of niacin. Control animals receiving the same basal diet with the addition of niacin showed no anaemia. It was concluded that niacin is necessary for normal erythropoiesis in the pig. In young growing dogs, Elvehjem's colleagues (234) observed that niacin was only partially effective in counteracti g the black-tongue syndrome produced by feeding a niacin-free basal ration containing 1 per cent sulphasuccidine. Pteroylglutamic acid helped to produce more consistent responses to niacin therapy, but the anaemia, which at this time was macrocytic, remained unaffected. Complete responses in haemoglobin and in growth were obtained when liver extracts were given in conjunction with niacin and PGA. When the protein level of the diet was increased from 19 to 24 or 30 per cent, the need for PGA could no longer be demonstrated. This functional relationship between PGA, niacin, and liver extracts in the treatment of the niacin-deficiency syndrome in dogs is

interesting and has also been observed by Sebrell (244) in dogs on the Goldberger diet number 123 given 50 mg. of indole daily to produce severe anaemia.

Amino acids.—The occurrence of dyshaemopoiesis in protein deficiency is well known, but the part played by the various amino acids individually and in combinations both in man and in lower animals has still to be clarified. Hall, Bowles & Sydenstricker (114) studied the blood changes in rats which were maintained on diets devoid of one of the indispensable amino acids. In phenylalanine deficiency, there was a reduction of blood volume and of circulating reticulocytes, although blood counts and plasma protein levels remained normal. In deficiency of valine or of isoleucine, reductions in blood volume, plasma protein, and reticulocyte count were observed. Sebrell (244) investigated the effect of dietary restriction of each one of the ten essential amino acids on the blood regeneration following haemorrhage in young albino rats on a protein-free diet. It was found that the most profound depression of blood formation occurred with diets deficient in valine or histidine and that a dietary deficiency of any one of the essential amino acids, with the exception of arginine, caused some interference with blood regeneration.

In dogs rendered anaemic and hypoproteinaemic by feeding a low-protein diet and by frequent bleeding, Robscheit-Robbins & Whipple (230) investigated the relative capacities of various dietary proteins to build up haemoglobin and plasma protein. The potencies of fresh and processed meat, different egg fractions, fresh ox heart, and canned salmon muscle were observed. Meat produced the highest new total protein in blood and more haemoglobin than plasma protein, whereas egg favoured the production of plasma protein. Ox heart and salmon muscle resembled meat, but were less effective. In further studies the authors (231) reported results with milk products and wheat and groundnut flours as compared with liver. Casein, either purified or commercial, was the most effective protein for production of haemoglobin and of plasma protein, whereas groundnut flour was less than half as effective as casein. Wheat flour produced a good response but was unpalatable.

Altmann & Murray (7) studied the blood picture of 34 children suffering from "malignant malnutrition." In 26 cases the anaemia was normocytic, in four microcytic and in four macrocytic. The improvement of the anaemia on treatment with skim milk powder alone prompted the authors to advance the hypothesis that the anaemia of "malignant malnutrition" is due to lack of protein or some specific amino acid. Bénard *et al.* (20) reported a case of severe orthochromic anaemia in a man of 70 in whom liver therapy produced only slight improvement, but methionine, 3 gm. daily, produced progressive improvement.

Antagonists of pteroylglutamic acid.—The literature on pteroylglutamates is steadily growing. It is now well established that although pteroylglutamic acid has an undoubted effect in transforming megaloblastic marrow to a normoblastic one, it has no protective influence on the neurological

lesions of pernicious anaemia (134). The activity of conjugates of PGA in pernicious anaemia was investigated by Wilkinson & Israëls (297), who found that synthetic pteroyl-tri- and -di-glutamic acids administered by oral or parenteral route were capable of inducing haematological remission. Swendseid *et al.* (266) demonstrated that crystalline pteroyl-heptaglutamate given orally or parenterally to sulpha-treated rats resulted in an increase in the granulocyte count similar to that obtained with equivalent amounts of PGA.

One of the most important developments in connection with pteroylglutamates is the synthesis of antagonists. These compounds are used currently in experimental procedures to study the functional relationship between PGA and the anti-pernicious-anaemia principle of the liver and as therapeutic agents in leukaemia and other neoplastic diseases. Minnich & Moore (199) studied the acute effects produced by large doses of 4-amino-PGA in guinea-pigs; profound depression of the marrow resulting in normocytic anaemia, granulocytopenia, and thrombocytopenia was observed. In a later study, using smaller doses of various antagonists in order to produce chronic changes, Innes *et al.* (132) found 4-amino-PGA to be the most active compound in depressing haemopoiesis. The anaemia was normocytic and normochromic, and no true megaloblasts were encountered in the marrow. Recovery ensued within 24 hr. of stopping the drug. In dogs, Thiersch & Phillips (269), using large doses of aminopterin, observed diarrhoea, leucopenia, and depletion of the marrow with the appearance in the marrow of megaloblasts, giant metamyelocytes and hypersegmented polymorphs. In pigs on a purified synthetic diet containing 0.2 per cent of PGA-antagonist, Wintrobe (54) reported the production of severe macrocytic anaemia, leucopenia, thrombocytopenia, and megaloblastic change of the marrow. Treatment with PGA produced a good response but thymine and xanthopterin were found to be inactive; purified liver extract and crystalline vitamin B_{12} had only a slight effect. Ritter & Oleson (229) observed marked degeneration of the small lymphocytes in the spleen and thymus, accompanied by progressive aplasia and fibrosis of the cellular elements of the marrow in young rats given aminopterin. Higgins & Woods (123) observed that adrenalectomy considerably minimised the changes which the analogues induced in the spleen, thymus gland, peripheral blood, and bone marrow of normal adult rats.

Following the observations of Little and his co-workers (179) that antagonists of PGA can prevent the growth of Rous Sarcoma in chicks, and of Farber *et al.* (89) that pteroyl-tri- or -di-glutamic acids when given to children with leukaemia appeared to accelerate the disease process, the use of these antagonists in the treatment of leukaemia is being actively explored. Of 43 leukaemic patients treated with aminopterin by Meyer *et al.* (197) only four showed improvement, 15 showed toxic effects necessitating discontinuance of treatment, and in the remaining 24 the course of the disease was unaffected. Berman *et al.* (25) treated nine patients with chronic leukaemia who showed no subjective improvement, although a reduction in the

stem cells, granulocytes, and immature lymphocytes was obtained. Disturbance of erythropoiesis characterised by megaloblasts in the marrow was also observed. Farber (88) records clinical improvement in more than half of 60 children with acute leukaemia treated with the antagonists aminopterin, "amethopterin," or "amino-an-fol." Dameshek (65) reports that of 34 cases of acute or subacute leukaemia, mostly in adults, treated with aminopterin or other PGA-antagonists, a third showed clinical and haematological remission lasting at least two months and up to eight and a half months. Remissions were obtained more commonly in the lymphoblastic type, and least often in the monocytic type. Stickney *et al.* (261) treated 21 children with acute lymphatic leukaemia and 33 adults with myeloid, lymphatic, normocytic, or undefined leukaemias with aminopterin. In five of the children and three of the adults complete remissions of the clinical condition and character of blood and bone marrow lasting up to four months were secured. Thus, as Dameshek (65) pointed out, the proliferative phase of the leukaemic process is by no means cured by the antagonists, although temporary remissions have been brought about by their use. The most pressing problem is the prevention of toxic manifestations due to the drug and further research for less toxic compounds is indicated. In this connection may be mentioned the disappointing results obtained by Gellhorn & Jones (100) in treating cases of lymphosarcoma and acute leukaemia with another antivitamin, desoxypyridoxine. The trial was based on the fact that lymphosarcoma transplants would not take in mice fed on a pyridoxine-deficient diet.

Malnutriture and Hepatic Function

Nutritional factors in hepatic injury.—In an admirable monograph, Himsworth (125) summarised our knowledge of the experimental and clinical aspects of the nutritional approach to liver disease. The subject was discussed at the International Biochemical Congress, and, as an introduction to what follows, a very brief summary may be given. Dietary deficiency can produce two separate types of hepatic injury, and each ends in fibrosis. One type of nutritional injury produces the diffuse fine fibrosis such as is found in Laennec's or "alcoholic" cirrhosis; it is usually preceded by fatty infiltration and can be caused by diets rich in fat and deficient in lipotropic factors such as choline or methionine, or by depleting the body of methyl groups. Gross dietary deficiency, especially of protein, can produce it in man, and it is believed that "alcoholic" cirrhosis is largely the result of malnutrition and not of a direct toxic action of alcohol. The second type is massive necrosis following an intense swelling of the liver parenchyma; if the animal survives there is nodular hyperplasia. This type can be caused by diets deficient in cystine and vitamin E which may act through a detoxifying mechanism. In both types the lesion may be secondary to interference with blood-supply and lymph-drainage through fatty infiltration or cellular swelling.

The rôle of alcohol in the production of fatty infiltration and cirrhosis of the liver in rats was reported in an interesting and well-designed study by

Best *et al.* (30). Fatty infiltration and consequent fibrosis, closely resembling the lesion in the chronic alcoholic, were seen in these animals and could be prevented entirely by increasing the intake of lipotropic agents either as choline, methionine, or casein. Dietary supplements of sucrose caused similar hepatic lesions to those produced by isocaloric amounts of ethyl alcohol. The experiments show that so far as the liver is concerned, alcohol is no more toxic than sugar and that imbalance between caloric intake and lipotropic factors is the cause of the liver lesions. Best suggests fortification of alcoholic beverages with lipotropic agents, but points out that this raises "scientific, moral, financial and gustatory problems." They should be raised and conquered.

The ability of dietary constituents to influence the rate of removal of collagen laid down in the liver of rats during the administration of hepatotoxic agents was studied by Morrione (201), who observed that a low protein diet supplemented with methionine, choline, and cystine produced the greatest decrease in collagen but that recovery was impaired by a low-protein high-fat diet. Ligation of the portal vein retarded recovery unless hepatic adhesions were present.

Doubt still exists in animal experiments as to whether hepatic cirrhosis occurs as a sequel to fatty infiltration without the supervention of actual necrosis of liver cells. Sellars (245) observed no parenchymal necrosis in the development of hepatic cirrhosis in rats produced by feeding hypolipotropic diets; Glynn, Himsworth & Lindan (105) also reported the production of diffuse hepatic cirrhosis ("portal cirrhosis") as a consequence of fatty infiltration in rats without necrosis. György & Goldblatt (108), in their studies extending over many years, also observed diffuse hepatic fibrosis in rats maintained on diets low in lipotropic factors; with lard and codliver oil as a source of fat, the incidence of cirrhosis was greater than with vegetable fat. They also found that tocopherol prevented the development of dietary massive or zonal necrosis and compensated for the absence of cystine or methionine. Wahi (288) found that on low-protein, low-fat, high-carbohydrate diets, young rats developed necrotic liver lesions whereas adult animals developed fatty infiltration followed by incipient fibrosis.

György, Rose & Shipley (109) found that oral administration of oestrone, oestradiol benzoate, and ethinyl oestradiol at levels of 30 μg. daily produced lipotropic action, the effect being enhanced by addition of methionine; progesterone, desoxycorticosterone acetate and testosterone exerted no lipotropic effect. Glynn *et al.* (105) refer to a significant lipotropic effect of "liver extract." The lipotropic action of lipocaic on a diet high in fat and low in protein is due to the choline present (296); and inositol does not exert its lipotropic effect in the liver of rats by stimulating the turn-over of phosphatides, whether choline-containing or not (34).

The progressive changes in liver composition, bromsulphthalein clearance, blood and plasma volumes, thiocyanate space, total plasma nitrogen, and liver cytology in rats fed protein-free diets containing low and large amounts of choline were studied by Wang, Hegsted & co-workers (289).

Early in the period of deficiency, the liver showed considerable loss of protein and accumulation of fat and glycogen, the fatty infiltration being present even in the high-choline group. An interesting feature was "a return toward normality in liver structure both chemically and histologically" during the later periods of depletion.

Brief mention may be made in this connection of the important studies of Hartroft & Best (119) on the relation of choline to renal lesions. By subjecting young albino rats to choline-deficient diets for short periods initially and then maintaining them on normal diets, the authors observed definite arterial hypertension, cardiac hypertrophy, and severe renal damage characterised by a reduction in the glomerular capillary bed. The authors point out that "there is no apparent immediate clinical application of these findings."

In the light of the newly acquired experimental evidence, the treatment of liver disease with dietary factors is being continued actively. At the same time, efforts are being made for the early detection of hepatic injury by the simultaneous and more frequent use of liver biopsy and liver-function tests. The effectiveness of new therapeutic measures in the treatment of cirrhosis of the liver has been reviewed (56), and there is no doubt of the importance of a nutritious diet in the therapeutic regimen of cirrhosis (211). A significant development during the past year has been the demonstration of diminished capacity of patients with decompensated cirrhosis to excrete sodium and of the beneficial effect of low sodium diets in clearing the oedema and ascites (86). This finds support in the observations (185) that in dogs with experimentally-induced ascites, high-protein, low-sodium diets produce minimum fluid accumulation. Another advance, especially in cirrhosis with gastrointestinal disorders, is the use of intravenous amino acid mixtures and concentrated human serum albumin (83, 87, 165). Although the use of intravenous human serum albumin had no definite beneficial effects and was not free from danger (87), the recent controlled observations of Eckhardt, Faloon & Davidson (83) tend to show that patients with advanced cirrhosis are able to tolerate the amino acids well and were benefited by the treatment.

The electrophoretic pattern of the plasma proteins in nine patients with hepatic cirrhosis was studied by Martin (190): a gross increase in the γ-globulin fraction and a marked decrease in the albumin fraction were present in all cases. Paul *et al.* (213) investigated the protein components by electrophoresis of simultaneously collected samples of plasma and ascitic fluid in several cirrhotic patients over periods ranging from seven weeks to six months. Ascitic fluid was found to be richer than plasma in albumin, α_1-globulin, and γ-globulin, while plasma was richer in α_2- globulin, β-globulin, and fibrinogen. It may here be noted that Keys and his colleagues (268) have shown that there are no changes in the electrophoretic diagram that are specifically characteristic of caloric undernutrition, whether produced by complete starvation or semi-starvation. Kinsell *et al.* (148) found that the rate of removal of intravenously injected methionine from the blood was significantly slower in patients with hepatitis and hepatic fibrosis than in normal individuals. Abnormally high concentrations of amino-nitrogen in

the urine of a case of hepatolenticular degeneration (Wilson's disease) when on a diet low in protein was reported (282). The data suggest that hepatolenticular degeneration presents a fundamental defect in amino acid metabolism.

Franklin, Popper & co-workers (95) have reported studies on the relation of liver-function tests to liver histology, made possible by the increasing use of the liver-biopsy technique. A correlation was established between definite histological features and the function tests by appropriate statistical methods. The most significant correlation was found between diffuse hepatic cell damage and the albumin/globulin ratio, cephalincholesterol flocculation, thymol turbidity, and bromsulphthalein tests. Kinsell *et al.* (149), in a similar investigation, attempted to correlate biopsy and biochemical findings with the activity, extent, and duration of the disease process.. Hillman (124) reported that the determination of plasma vitamin A and blood sugar responses to injection of epinephrine cannot be employed as a test of liver function; the latter finding may be contrasted with the observations of Kinsell *et al.* (150).

Clinico-pathological observations on cirrhosis of the liver in infants were reported by several authors (73, 143, 264) of whom Dekker-Jonker (73) suggests that cirrhosis of the liver may develop in intra-uterine life because of maternal malnutrition. Gentili & Tangheroni (101) believe that hepatic insufficiency is the most important factor in the toxaemia of gastroenteritis in infants and claim favourable results from the administration of choline chloride.

Before closing this section, special mention must be made of a classical morphological study by Gillman & Gillman (104) of a type of liver reaction characterised by the appearance of fat-free, glycogen-free vacuoles in the liver cells and of its relation to various forms of anoxia and malnutrition.

Kwashiorkor.—In recent years considerable interest has developed around a syndrome of malnutriture in childhood, originally described by Williams (298) as occurring in the Gold Coast under the African tribal name Kwashiorkor; synonyms are Infantile Pellagra and Malignant Malnutrition. The condition has since been described in many tropical and subtropical countries and has been adequately reviewed by Trowell (276). It commences after weaning and is characterised by failure of growth, oedema, hypoproteinaemia, gastrointestinal defect, anaemia, dermatosis, and a fatty liver, and it is attended by a high mortality. The aetiology of the condition is still obscure; Trowell (276) concludes that shortage of first-class protein of animal origin together with excess of carbohydrate is the most significant aetiological factor. Lehmann (174) suggests that tropical helminthic infestations can cause or accentuate certain features of the condition, such as the anaemia and pigmentary changes of the skin and hair. Gillman & Gillman (103) considered fatty infiltration of the liver as the essential lesion in Kwashiorkor and regarded the condition as merely one of pellagra of infancy. A functional failure of the liver cells to store glycogen when presented with abundant amounts of glucose has been shown by Holmes & Trowell (128) by chemical analysis of liver-biopsy specimens of cases of malignant malnutrition. Davies (67)

finds that acinar atrophy and fibrosis of the pancreas are primary and more constant changes in Kwashiorkor than those in the liver.

Most workers agree that vitamin therapy is disappointing. Gillman & Gillman (103) reported excellent results with dried stomach, but Trowell (276) was unable to confirm this finding. Altmann (6) and Trowell (276) report successful treatment of their cases with milk.

One of the puzzling features of Kwashiorkor had been its rarity among the undernourished peoples of Asia. Ramalingaswami *et al.* (219) have recently described typical cases from South India and it can no longer be doubted that Kwashiorkor occurs in that part of the world.

Nutritional gynaecomastia.—The association of malnutrition with gynaecomastia has received considerable attention. It is known that deficiency of the vitamin B complex and of lipotropic factors can cause gynaecomastia, and it is reasonable to assume that they act through the liver, since impaired liver function is a cause (29, 153, 180) apparently through its inability to inactivate oestrogens in the normal way. If chronic inanition causes decreased production of oestrogen, perhaps through a decrease in gonadotropic content of the pituitary gland (227), gynaecomastia might be expected when an adequate diet immediately follows prolonged undernourishment which, by accompanying deficiency of lipotropic and other factors, had caused impaired liver function. Korting (155) described 16 cases amongst 600 interned men observed during three years, but came to no conclusions apart from the great significance of "psychosomatic influences"; Kuhnke (163) saw a number of cases in Germany following the war but, unlike Bansi (15), did not associate them with undernutrition. Jacobs (136) reported a large series of cases amongst U.S. men in Japanese prisoner-of-war camps; the gynaecomastia appeared in 10 per cent a few weeks following an adequate diet (mainly Red Cross parcels) after two years of malnutrition; it then disappeared during a further 16 months of malnutrition, and appeared in nearly 50 per cent of the prisoners after liberation. Jacobs believes that the almost complete dietary absence of cholesterol and ergosterol, which he calls "essential steroids in the normal diet," "may have played an important part in diminishing the estrogenic and androgenic functions"; there is no evidence for this view and considerable evidence against it. Similarly Kyle (166) reported a man of 24 years with chronic ulcerative colitis, liver disease, and diabetes mellitus in whom gynaecomastia appeared not when the liver disease was most severe but later when he was eating freely, gaining weight, but neglecting his vitamin intake; it is claimed that "evidence is presented that the breast changes were probably due to a relative vitamin B deficiency." Davies (68) calls attention to the high incidence of gynaecomastia, breast carcinoma in males, and primary liver carcinoma in Africa, and suggests that "owing to the widespread malnutrition and consequent liver damage, Africans are subject to wholesale oestrinization."

We saw no cases of gynaecomastia either during the acute famine in the Netherlands in April and May of 1945, or immediately after the famine when calorie intakes were very high, and the acute caloric deficiency of the

famine did not cause liver damage; but we saw a number of cases in Germany where there was, in 1945, some deficiency of vitamins of the B complex and possibly of lipotropic factors and considerably higher calorie levels than in the Netherlands famine. It may be concluded from the evidence so far available that nutritional gynaecomastia may occur if there is liver damage from a malnutriture that is not sufficiently serious to cause marked decrease in the production of oestrogens.

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MUSCLE[1]

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From the functional point of view, the biochemistry of muscle is an advanced field, so much so that it cannot be considered without referring to biophysical and general physiological investigations. This is emphasised by the very valuable review of Sandow (1) which coordinates present biochemical research with the physiology of muscle function. The present reviewer will, while selecting investigations closely related to his personal interest, follow the same considerations. This seems important in order to avoid the deterioration of the review into a catalogue of recent papers.

Although the last review on muscle biochemistry appeared in 1942, it was not thought worth while to go back as far as that. The more important facts were in the meantime recorded under other headings. It is planned to present relevant data on the properties of muscle proteins, the processes probably involved in muscle contraction, recent biophysical investigations and the efforts continually made to explain the latter on the ground of the former. Reviews on certain parts of these problems are abundant (1 to 8).

Proteins

Structural proteins.—Three new proteins have recently been isolated from skeletal muscle which fall into this class. The detailed account of Bailey's work on tropomyosin has of late appeared (9, 10, 11). Tropomyosin has been isolated from alcohol-ether dried rabbit muscle and can be obtained in crystalline form. Its amino acid composition resembles that of myosin and on this basis Bailey considers tropomyosin a subunit in the building up of myosin. However, the rejuvenation of myosin being a slow process, it is surprising to find 0.5 gm. tropomyosin in 100 gm. fresh muscle. Solutions of tropomyosin in distilled water are very viscous; their viscosity is decreased by the addition of salt and is quite low at 0.1 *M* KCl. In viscous solutions of tropomyosin uniform fibres of 200 to 300 Å diameter are revealed by electron microscopy, whereas no structure can be observed in salt solutions. The x-ray diagram suggests that the fibres are produced on dialysis by the side to side aggregation of elongated molecules containing two parallel polypeptide chains.

From peptic digests of beef and horse muscle, Bourdillon (12) isolated a pepsin-resistant protein named peptomyosin, in crystalline form. It is heat and acid stable, and very viscous in distilled water; its molecular weight is 91,000, the same as that of tropoymosin. Actually, the properties of the two proteins are uncommonly alike. The significance of either protein is as yet unknown.

[1] This review covers the period approximately from 1946 to September 30, 1949.

The *N* protein of Gerendás & Matoltsy (13) has also been further studied. By its composition it appears to be a lipid-protein-nucleic acid complex (14). The isolated crystals show negative birefringence. *N* protein thus possesses the properties of the substance observed in the I band which extinguishes the positive double refraction of the actomyosin component.

The crystallisation of myosin by Szent-Györgyi (15) has undoubtedly helped us to arrive at a better understanding of this highly interesting protein. Its enzymatic properties will be discussed further in a later section (p. 377). The electrophoretic experiments performed with impure myosin (16) have revealed the presence of several components, but the study of crystalline myosin (17) shows it to be essentially monodisperse. From ultracentrifugal data, the molecular weight of crystalline myosin appears to be 1,500,000 (extrapolated). In the presence of concentrated urea, myosin breaks into fragments. While after a short treatment with urea, recombination of these fragments is observed, prolonged treatment leads to irreversible changes. But, in either case, myosin becomes polydisperse. Recent pictures by electron microscopy show myosin to consist of fibrils, with a tendency to form a network of anastomosing fibres. The myosin crystals are of paracrystalline nature; they are composed of very long fibrils (18, 19).

Erdös & Snellman (20) find the isoelectric point of myosin to be pH 5.4. This is only true in the presence of potassium chloride. When electrophoresis is carried out in the presence of calcium or magnesium ions, no isoelectric point is found throughout the range of pH 2 to 9. The mobility decreases in an alkaline medium to a low constant value and even at the highest pH investigated the protein migrates towards the negative electrode. Obviously, doubly charged ions are bound specifically to myosin and this in turn has an enormous influence on the charge of the protein, thus producing this unique effect. In a mixture of potassium and calcium salts, myosin migrates either to the anode or to the cathode, according to the ratio of the concentrations of the two ions. No migration was observed, when the ionic strength of both ions was equal (0.3μ).

Considerable attention has been given to the sulfhydryl groups of myosin. Bailey & Perry (21) have presented evidence that the destruction of some sulfhydryl groups in myosin destroys its capacity to split adenosinetriphosphate (ATP) or to bind actin. Native myosin contains 1.6 to 1.8 per cent apparent cysteine equivalents when titrated with iodine. Bailey & Perry found that actomyosin formation and the adenosinetriphosphatase activity of myosin were inhibited to the same degree, when myosin was treated with varying concentrations of iodosobenzoate, peroxide, iodine, *p*-chloromercuribenzoate or iodoacetamide. It seems, therefore, that some specific sulfhydryl groups are directly responsible for the binding of ATP and actin. This problem will be discussed further (p. 374).

Whereas myosin is a determining factor in the colloidal behaviour of actomyosin, actin shows a number of uncommon properties, which may have significance in the function of actomyosin. Feuer *et al.* (22) have

studied some details of the effect of inorganic ions on the polymerisation of actin. It is remarkable that the formation of thread-like molecules from small globular units proceeds at an optimum rate under the influence of physiological salt concentrations. Magnesium has an enhancing effect and it is suggested that an intermediate step of the process of polymerisation is catalysed by magnesium ions. Calcium ions, when used alone, cause rapid polymerisation whereas the process is slowed down to a minimum if calcium and sodium (or potassium) ions are added together in a concentration near that occurring in muscle. This is a clear case of ion antagonism observed in a simplified, dissolved system, *in vitro*, in a process which can be expected to play a role in muscle contraction. In accordance with these observations, some drugs were found to influence the rate of polymerisation: veratrine, quinine, and strychnine exerted an inhibitory effect; epinephrine displayed an accelerating effect, but only at certain definite calcium: potassium ratios. These results (23, 24) are interesting but they do not yet explain the mode of action of these substances, because the concentrations used were above the pharmacologically active levels.

After a preliminary report on the prosthetic group of actin (22), Straub & Feuer have now demonstrated (25) that actin contains ATP. When this ATP is removed, actin becomes inactivated: it does not polymerise any more and cannot form actomyosin. The ATP content of actin preparations varies from 1 to 2 per cent. ATP is present in a bound form, inaccessible to enzymes which utilise ATP. When actin is denatured by heat the protein is not precipitated, but its ATP is quantitatively released. Actin solutions are inactivated by dialysis, and the inactivation can be prevented by adding 0.004 per cent ATP to the outer fluid. In this way the reversibility of the polymerisation of actin is demonstrated.

ATP is actually the prosthetic group of actin, taking part in the specific change of the latter; during polymerisation some or all of the ATP contained in it is converted to adenosinediphosphate (ADP). At the same time, an equivalent amount of inorganic phosphate is liberated. The process is reversible: depolymerisation is connected with the taking up of inorganic phosphate and formation of ATP in the actin molecule. This is a case of mechanochemical coupling, in analogy to coupled phosporylations, the change of form of the protein molecule being the one component and the breakdown or synthesis of ATP the other.

In agreement with the above findings, it is observed that part of the adenosinepyrophosphates of muscle behaves in a way differing from the rest, i.e., it is not all split by the enzymes of muscle. Not only muscle, but heart, liver, kidney, etc., also contain protein-bound ADP or ATP (26, 27).

X-ray diagrams of polymerised (F) actin films were studied by Astbury (28, 29) and found to give reflections, which were formerly known to occur in Bear's Type-II fibrils (30). According to Astbury the "full muscle diagram is effectively the sum of the diagram given by extracted myosin and extracted F actin, in other words, it corresponds to the actomyosin complex."

Electron microscope studies of actin have confirmed the view (31) advanced by the study of viscosity and double refraction of flow, that globular (G) actin polymerises into fibrous (F) actin on addition of salts (18, 19, 28). It appears, as judged from the extreme regularity of the dimensions of fibrous actin, that the globular molecules are united by specific forces to form fibrils.

Actin is not yet known in a pure form, but its enzyme impurities, e.g., creatine phosphopherase, can be separated from actin by simple precipitation (32).

The properties of actomyosin have also been studied with modern methods. Although the discoverers of actomyosin (2) have suggested that myosin is able, up to a limit, in any ratio to bind actin, recent ultracentrifugal data show that the reaction is essentially stoichoimetric (33). When increasing amounts of actin are added to myosin in the ultracentrifuge, the component corresponding to crystalline myosin is seen to diminish and another component corresponding to actomyosin appears in progressively higher concentrations. At a ratio of 1 part of actin to 2.5 to 3 parts of myosin, all free myosin disappears and, on further addition of actin, a new component corresponding to free actin becomes visible. The above ratio is the one found by Balenovič & Straub (34) to occur in rabbit's skeletal muscle and, at the same time it is the actomyosin which shows the maximal drop in viscosity on addition of pyrophosphates (31).

The nature of the reaction between myosin and actin to form actomyosin in the absence of pyrophosphates is not yet clarified. Although myosin is unable, after the removal of some sulfhydryl groups, to bind actin any more, the assumption of Bailey & Perry (21) that actin is bound to the sulfhydryl groups of myosin and that the same sulfhydryl groups are responsible for the binding of ATP to myosin, seems to be an oversimplification. Generally, too much is at present put to the account of sulfhydryl groups. The appearance or disappearance of sulfhydryl groups is one aspect of protein architecture which is most easily determined, but there can be no doubt that other processes, not easily followed at present, go on at the same time. It is conceivable that a structural feature of the protein surface collapses when neighbouring sulfhydryl groups are blocked by a reagent and this will prevent another reactive group, which might be anything else, from reacting with actin.

Godeaux (35, 36, 37) has studied the effect of some sulfhydryl reagents on the syneresis of actomyosin threads. Titrating with ferricyanide, he found 0.82 per cent cysteine equivalents of reacting sulfhydryl groups, 40 per cent of which were blocked, while the rest remained free. When the latter groups were bound by the sulfhydryl reagent syneresis was hardly affected; it was, however, abolished when the last few sulfhydryl groups had been destroyed and before the blocked ones were involved. Szönyi has observed in the reviewer's laboratory (38) that the adenosinetriphosphatase activity of actomyosin is nearly completely inhibited by *p*-chloromercuribenzoate, iodosobenzoate or ferricyanide in concentrations which have practically no

disintegrating action on the actomyosin complex. These results are in contrast to Bailey & Perry's theory.

Actin and myosin, derived from various organs and species, react with one another equally well, without any sign of specificity (39 to 42). The myosin and actin content of uterus show significant changes during pregnancy (43). In the nonpregnant uterus, the actin: myosin ratio is far below that of skeletal muscle. Accordingly, threads prepared from such extracts show only a feeble reaction with ATP. While the amounts of both substances (per gm. uterus) increase during pregnancy the rise is more pronounced in the case of actin, so that, in late pregnancy, the actin: myosin ratio reaches the value found in skeletal muscle. Threads prepared from such extracts "contract" violently on addition of ATP.

Similar studies on heart muscle show that the extractable actomyosin of the left ventricle contains relatively more actin than does the actomyosin from the right ventricle (44).

It has been shown by Erdös (45) that the disappearance of ATP, the decrease of the solubility of myosin and the increase of rigidity are interrelated during the development of *rigor mortis*. Further it was emphasised that the presence of ATP is an important factor for the extraction of myosin. Bate-Smith & Bendall (46) have confirmed these results. Erdös has further shown that the addition of ATP to the extracting fluid increases the amount of myosin going into solution. It has been suggested that the Deuticke effect may be due to the reduction of ATP content during fatigue. Dubuisson (47) has, however, found that ATP merely accelerates the rate of extraction of myosin but does not increase the amount extracted.

Electrophoretic studies on the protein extracts obtained from muscle with concentrated salt solutions, were made by Dubuisson (48.) Three myosin components were characterised, α, β and γ. Of these, α seems to correspond to actomyosin and β to crystalline myosin, whereas nothing definite is known of the γ component. Hamoir (49) has described the separation of α and β myosin. Lately, Dubuisson (50) has discovered a hitherto unknown protein, named contractin, in the electrophoretic study of contracing muscles. The amount of contractin, appearing in solution at $\mu = 0.15$, seems to be proportional to the degree of contraction.

The amount of myosin extractable from muscle decreases during denervation atrophy (51) and, simultaneously, a decrease in the myogen and an increase in the myoalbumin fraction is observed (52). The actomyosin obtained from newborn animals has a much lower actin content than that of adults (53).

Water-soluble proteins.—A detailed study of the electrophoretic behaviour of muscle extracts obtained at physiological ionic strength, established the presence of eight separate components (54). Distèche was able to characterise eight different components in similar extracts through the solubility curve obtained at constant ionic strength and varying pH. Some of these can be identified with the components observed in electrophoresis. Three of the components have been crystallised by this simple method at the appro-

priate pH and one of these seems to be identical with aldolase, although its activity is not maximal (55). In this respect it resembles the myogen A crystals of Baranowski (61).

Cori's laboratory is very actively engaged in the characterisation of the enzymes involved in muscle glycogenolysis. After the isolation of phosphorylases, four other enzymes have been obtained in crystalline form from rabbit muscle extracts: aldolase (56), glyceraldehyde phosphate dehydrogenase (57), glycerophosphate dehydrogenase (58) and phosphoglucomutase(59, cf. 60). During the course of this work, it turned out that the electrophoretically homogeneous myogen A, earlier isolated in a crystalline form by Baranowski, is a mixture of proteins from which glycerophosphate dehydrogenase can be isolated in a pure form. In spite of the initially high aldolase content of crystalline myogen A, pure aldolase cannot be prepared from it (61).

Petrova has purified amyloisomerase (62), the enzyme which synthesises the 1,6 linkages necessary for the formation of glycogen. The same enzyme has been named isophosphorylase by others (63, 64). While the enzyme synthesises no glycogen, when used alone, it helps, in the presence of phosphorylase, to build up a polysaccharide which gives a red colouration with iodine.

Arginine-ATP phosphopherase has been prepared in two crystalline forms by Szörényi *et al.* (65). Phosphopherase A contains manganese, is inactivated at 50°C, remains active on dialysis and crystallises in bipyramids. Phosphopherase B crystallises in cubes, contains no alkaline earth metal, and is activated by manganese or magnesium ions, the protein alone being inactive.

The mode of action of phosphoglucomutase seems to have been cleared up by the independent work of three laboratories (66, 67, 68). Glucose-1,6-diphosphate is the prosthetic group of the enzyme. The reaction can be formulated in the following way:

glucose-1-phosphate + glucose-1,6-diphosphate ⇄ glucose-1,6-diphosphate + glucose-6-phosphate

This equation is elegantly proved by using radiophosphorus as an indicator (68). Following up the clue that the coenzyme of phosphoglucomutase is formed at the time of formation of fructose-1,6-diphosphate, a new enzyme reaction was observed in muscle extracts:

glucose-1-phosphate + ATP ⇄ glucose-1,6-diphosphate + ADP

The enzyme catalysing this reaction is thus an analogue of (but not identical with) hexokinase. The new enzyme is activated by magnesium and manganese ions; it has been partially purified (69).

Adenosinetriphosphatase

The enzyme activity of myosin has challenged the imagination of many workers, but, despite many investigations, nothing but the shape of the problem has emerged; myosin is responsible for the adenosinetriphosphatase

and adenylic acid deaminase activity of the water-insoluble fraction of muscle.

In this ATP era of biochemistry it is worth while to recall recent improvements in the preparation of ATP (70 to 73). The need of new, more specific, methods for the determination of ATP is also felt, since the occurrence of metaphosphates and labile phosphate in nucleic acids is known. The method of Borbiró & Szent-Györgyi (27) renders the determination of P_7 possible even in the presence of excessive amounts of inorganic phosphate. Another new method (75) is based on the specific reaction of ATP with actomyosin.

There is no other enzyme known that splits off only one inorganic phosphate residue from ATP except myosin. Accordingly, myosin is an adenosinetriphosphatase and not an apyrase. The latter name should be reserved for, and applied to, enzymes which split off both labile phosphates. In muscle, the second labile phosphate is split off by the intervention of myokinase. This specific organisation of ATP breakdown is possibly related to the utilisation of the energy of ATP in muscle contraction.

Ten years have elapsed since the discovery of the adenosinetriphosphatase activity of myosin by Engelhardt & Lyubimova (76). Throughout this period all attempts to separate such an enzyme from myosin have failed. It may be possible to prepare a myosin free of adenosinetriphosphatase activity (77) and it is possible to prepare fractions from myosin which are somewhat more active than pure myosin (78), but the problem of separation is not settled and the evidence is rather poor. It has been shown in early works (2), and again confirmed by Biró & Szent-Györgyi (79), that myosin shows maximal activity in a special colloidal state, when it is completely precipitated. In such a heterogeneous system, the treatment of myosin with lanthanum salts which resulted, in the experiments of Polis & Meyerhof (78), in a two to threefold rise of activity, is by no means a proof of the separation of myosin and adenosinetriphosphatase. Evidently, a portion of the myosin molecule, and not all of it is responsible and necessary for the enzyme activity. At the moment, all evidence favours the view that the adenosinetriphosphatase activity is linked to myosin. The case of grasshopper myosin is rather an exception in that the greater part of the adenosinetriphosphatase activity can be washed out of it (80).

Hermann states (81, 82, 83) that the so-called Schmidt deaminase is identical with myosin in the same sense as adenosinetriphosphatase. It is very significant that the rate of renewal of adenine-amino nitrogen is quite high (74). The high adenylic acid deaminase activity of myosin was noticed earlier by Ferdman & Nechiporenko (84). Hermann has shown that all adenylic acid deaminase preparations contain myosin and the highest activity is observed when several times recrystallised myosin is used. On precipitating myosin with sodium sulfate at pH 5.3, a fraction was obtained in small quantities, which was two or three times more active than the original myosin. But, as in the case of lanthanum-fractionated adenosinetriphosphatase, further purification was not achieved and it must be admitted that

adenylic acid deaminase activity is linked with myosin, a part of the myosin molecule being responsible for this enzyme activity. The fact that this part of myosin is not the same as the one responsible for adenosinetriphosphatase activity is shown by the more selective blocking of the latter in presence of sulfhydryl reagents.

The further problem is, therefore, to know the role of the unity of these, and possibly other, enzymes within one fibrous molecule, rather than, whether these two enzymes can or cannot be separated from myosin.

The adenosinetriphosphatase activity of muscle is not wholly connected with myosin. Kielley & Meyerhof (85) have isolated an enzyme which is activated by magnesium and not by calcium ions. However, further purification of this enzyme is needed before its relation to myosin may be clarified, the process of purification described being no guarantee for the absence of myosin in the preparations used.

Zbarski & Brisker (86) describe the apyrase activity of liver, brain, and other organs. The activity is found mostly in the soluble fraction and is unrelated to the myosin type adenosinetriphosphatase. Epelbaum *et al.* (87) find that brain contains two fractions; the soluble adenosinetriphosphatase is activated by magnesium ions, the insoluble one by calcium ions.

Bravermann & Morgulis (88) have studied the activity of myosin as a function of the calcium:magnesium ratio and conclude that, in muscle, myosin is expected to split ATP at a reduced level of activity.

A warning against this approach to the problem comes from Steinbach (89). It seems that caution must be exercised when conclusions are drawn from the activation of an adenosinetriphosphatase by calcium ions. In frog muscle homogenates Steinbach finds no activation by calcium ions, either in the soluble or in the insoluble faction. Only when the residue is treated with a strong potassium chloride solution to extract myosin—when the sum total of enzyme activity diminishes—is the insoluble adenosinetriphosphatase activated by calcium ions.

The adenosinetriphosphatase activity of developing (90), vitamin E deficient (91) and atrophying muscle (51) was studied.

The role of myokinase seems now to be fairly well established, as former conflicting reports (2, 92) can be explained by assuming the function of adenosinetriphosphatase and myokinase alone, without involving a special mechanism of ADP-isomerisation (93). Having shown the presence of myokinase in liver, spleen, erythrocytes, etc., Kotelnikova proposes the more appropriate name of ADP-phosphomutase instead of myokinase (94).

Studies on the inhibition and activation of the adenosinetriphosphatase of myosin by a number of pharmacologically active substances have not yet yielded outstanding results. Torda & Wolff (95) described slight activation by acetylcholine. Singher & Millman (96) found no effect of acetylcholine on uterus myosin adenosinetriphosphatase. Binkley has however recently observed (97) that posterior pituitary gland extracts inhibit the enzyme. This inhibition is greatly reinforced by acetylcholine, although the inhibition can be observed only during the first few minutes of contact.

Metabolism

This is rather a neglected field recently. It is not intended to summarise current knowledge but to mention only some important results. The carbohydrate metabolism of the isolated rat diaphragm suspended in oxygenated Ringer's solution is a material widely used for the study of the effect of hormones *in vitro*. Stadie & Zapp (98) find an increase of glycogen formation on addition of insulin, but the effect is most pronounced in the absence of potassium ions. When the cations are replaced by potassium (to about 75 per cent), the action of insulin disappears. Türkischer & Wertheimer (99) come to similar conclusions regarding the effect of potassium ions, and find moreover, normal serum to inhibit the action of insulin. Epinephrine completely inhibits glycogen formation in the isolated rat diaphragm according to these authors. Cohen (100) finds no effect of epinephrine on the glucose utilisation. However, the anaerobic glycolysis was inhibited when the rats were injected with epinephrine 40 or 70 min. prior to the excision of the diaphragm. Desoxycorticosterone inhibits glycogen synthesis in the isolated diaphragm, according to Verzár & Wenner (101). The diaphragm of adrenalectomized animals behaves in the same way as those of normals, when insulin or desoxycorticosterone is added. Having confirmed these results, Li *et al.* (102) found that the diaphragms of hypophysectomised animals also behaved like those of normal animals.

The accumulation of glycine in muscle tissue was studied with the aid of the isolated rat diaphragm (103). Coupled with an exergonic process, glycine is concentrated 3 to 5 fold and the glycine content of the tissue follows the changes in the surrounding fluid. The uptake of glycine was found to be inhibited by other α-amino acids.

Among oxidative processes, the mechanism of oxidation of succinate is still one of the major problems as it reflects the general mechanism of oxidation. Slater (104) has presented new evidence for the occurrence of a factor that links succinic dehydrogenase to the cytochrome system. The study of succinoxidase in *C. diphtheriae* has raised the problem of the identity of cytochrome-*b* and succinic dehydrogenase (105).

Ball & Cooper (106) prepared succinoxidase by a new method in which phosphate buffer is dispensed with. In this way an unstable preparation is obtained, the deterioration of which is attributed to the action of a phosphatase, suggesting the probable role of some phosphate ester in succinate oxidation. The enzyme preparation contained 37 per cent lipid and 1.5 per cent total phosphorus. Of the latter, one-third was nucleic acid phosphorus and a small fraction was labile. The possible participation of flavin in succinate oxidation is again considered. It is interesting to mention in this connection that an isomer of riboflavin, *l*-lyxoflavin has been isolated from human myocardium (107).

The critical study of Keilin & Hartree (108) reveals the importance of the colloid state of heart muscle succinoxidase preparations. Inhibitions and activations can be mostly explained by a corresponding change in the colloidal behaviour. If a foreign protein is present and flocculated, this will

lead to activation. These observations invalidate the claims as to supposed intermediary links between succinic dehydrogenase and cytochrome-*c* (109, 110, 111) as the action of such substances is explained by the influence of the denatured protein they contain. The possibility of the existence of such a factor is however still open. Against the current belief (112) that calcium activation of succinoxidase is due to the removal of diphosphopyridine nucleotide, Keilin & Hartree maintain that calcium activation is observed in preparations devoid of the coenzyme, and attribute the phenomenon to the action of calcium on the colloidal particles.

The endogeneous respiration and the action of some respiratory inhibitors have been systematically studied on ràt heart slices (113).

Peptidases of muscle tissue were studied by Smith (114) and a specific carnosinase was found (115) in many tissues but not in muscle. Carnosine and anserine are present in muscle in a high and constant concentration and the ignorance of their function prompted a number of investigations. Phosphocarnosine was synthesised and shown to be rapidly hydrolysed by muscle tissue (116). Part of the carnosine is not dialysable and is most probably bound to proteins (117). Tydman-Chetverikova finds great sexual differences in the carnosine and anserine content of rabbit skeletal muscle (118). Whereas the sum of the two substances is nearly the same, males have a little more carnosine than anserine, and females have only one-fourth as much carnosine as anserine in their muscles. There is a sharp reduction in the concentration of both substances during starvation: carnosine nearly disappears and the anserine content is reduced by two-thirds. The effect of carnosine on carbohydrate metabolism was investigated by Severin *et al.* (119).

The Reactions Produced by ATP on the Proteins of Muscle, in Model Systems and Living Muscle

When ATP is added to actomyosin in high salt concentration, the viscosity of the fluid is significantly lowered. The effect was explained as a dissociation of actin and myosin (2). An alternative explanation, assuming the coiling up of (myosin) particles, has been proposed by Needham *et al.* (120) but received no support, although recent light-scattering studies of Jordan & Oster have revived this theory (121). The viscosity effect is not specific for ATP, similar effects being produced by inorganic pyrophosphate, calgon, and inorganic triphosphate, in a somewhat higher concentration (122). Less ATP is needed to produce the drop of viscosity in the presence of magnesium ions, and more ATP in the presence of calcium ions (122, 123). It should be noted that these ions have just the opposite effect on the adenosinetriphosphatase activity of myosin. That myosin takes up phosphate when treated with ATP, was first shown by Szörényi & Chepinoga (124). Lately, Buchtal *et al.* (125) have shown that myosin, treated with ATP and then washed several times, takes up adenine, ribose, and labile phosphate. Such a process has been several times suggested as a means of energisation of myosin, but further confirmation must be awaited. The presence of labile phosphate, adenine, and ribose in a number of enzymes has been recently demonstrated

(126); however, the significance of this finding is not yet fully comprehended.

The syneresis of actomyosin gels in low salt concentration ("thread contraction") has been very actively investigated. Threads prepared from actomyosin, suspended in salt solution of slightly lower concentration than the physiological one, shrink rapidly and even reversibly in all directions. This basic finding of Szent-Györgyi (2) has been amply confirmed and is still the driving force to explain the structural changes during muscle contraction in terms of actomyosin-ATP interaction.

The structural changes produced by ATP in the actomyosin gel were studied in the electron microscope by Perry *et al.* (127) and Snellmann & Erdös (19). There is hardly any doubt that the process induced by ATP is an intermolecular synersis. The extrusion of the greatest part of water present between the anastomosing fibres of actomyosin permits the close, side-to-side, aggregation of the fibres. As Astbury points out, if myosin and actin lie lengthways in the muscle structure syneresis cannot lead to shortening of the muscle. Szent-Györgyi (128) still maintains that a properly oriented actomyosin thread shows anisodiametrical contraction. His evidence is however, circumstantial and rests on earlier experiments of Gerendás (129). Threads of pure actomyosin are fragile and they cannot be stretched. Gerendás treated actomyosin with zinc ions. These zinc-treated threads were stretched and their anisodiametrical contraction was observed under the influence of ATP. Such threads are to some extent certainly denatured and this possibility rather weakens the argument of Szent-Györgyi. Threads made from undenatured actomyosin certainly contract only isodiametrically.

Engelhardt (130) was the first to notice the action of ATP on the colloidal behaviour of actomyosin. He found a lengthening of loaded myosin threads after treatment with ATP. The apparent disagreement has been removed by the study of Ivanov *et al.* (131, 132). They have found that the primary action of ATP on actomyosin always produces syneresis. In view of the nature of this reaction, loaded threads cannot shorten, but the gel will become elastic and lengthen.

In spite of the above controversy, there is no doubt that the phenomenon of syneresis ought to be of deep significance. It should be enough to point out that the reaction is entirely specific for ATP. Denaturing agents may produce lengthening of loaded threads, but they will not induce the shrinking of unloaded threads.

Szent-Györgyi has, in his latest study (128), developed some new methods for the study of isolated muscle strips. These are actually systems, systematically simplified with the view of studying the physiological role of actomyosin. Psoas strips soaked in glycerol undoubtedly contain actomyosin in a physiological state from the structural point of view, and they can be made to contract in ATP solutions. In Szent-Györgyi's view, the results of Varga (133), criticised by Hill (147) and Sandow (1), actually represent the mechanism underlying muscle contraction. The contracting units, "autones" are either in a contracted or in a relaxed state and the actual contraction depends on an all or none equilibrium reaction of these autones. Obviously

some important consequences of this theory come into collision with facts as presently known. Moreover, these new methods and fibre preparations have to be further characterised. Nevertheless, his view is strengthened by experiments on frozen muscle strips, which show, under certain conditions, a reversible tension-temperature change. In this simplified muscle system, which may be looked upon as an organised actomyosin, the maximum work done shows a linear dependence on temperature. Furthermore, the properties of this system reveal that its elasticity and the changes of elasticity in it are determined by the actomyosin and ATP they contain. According to Szent-Györgyi, the equilibrium of the autones is coupled with a regulatory system in living muscle. The latter is reversibly inhibited by chloroform. In fact, chloroform treatment of muscle, gives results which are in harmony with the conclusions of Varga (134) and differ from those obtained with untreated muscle strips.

Assuming that actomyosin which does not split ATP is still able to contract (135), Szent-Györgyi believes that the free energy of the actomyosin system can be divided in two portions: formation of myosin-ATP complex with disruption of formerly existing cohesive forces and, then, contraction. The second reaction is inhibited in muscle by the above mentioned regulatory mechanism. Excitation then means the physiological removal of this inhibition. It is to be emphasised that the splitting of ATP is not mentioned in this scheme. The free energy spent at the maximum temperature (just before heat denaturation sets in) is 11,000 cal. and it is inferred that just this much can be obtained by the splitting of one mole of ATP during recovery.

It is clear that ATP has a pronounced influence on the irritability of muscle. Babskii & Minaev (136) and Buchtal & Folkow (137) describe the increase of sensitivity of muscle to acetylcholine after the application of minute amounts of ATP. ADP has a similar action as ATP whereas adenylic acid is inactive. Surprisingly, when adenylic acid is applied together with pyrophosphate (but not with orthophosphate), sensitisation is observed (136, 138). No mechanism is known which would synthesise ATP or ADP from pyrophosphate. Sheikhon (139) has described the reversible increase of irritability of frog's skeletal muscle under the influence of very dilute ATP solutions and a transient excitatory effect on the isolated frog heart (140). Lichtneckert & Straub (141) found that 0.5 μg. ATP or ADP could restore the normal amplitude of a quinine treated heart, and this effect was lasting. The effect is so specific that it can be used for the semiquantitative identification of ATP and ADP. It was formerly reported by Rózsa (142) that less than one μg of ATP elicited contraction in isolated muscle strips.

The above experiments were earlier interpreted as an analogue of "thread contraction" assuming that the phenomenon was due to the action of ATP on the contractile system. But, apart from the problem of the permeability of the cell membrane to ATP, it is impossible to forget that, in all these cases, a several hundred times higher concentration of ATP is present inside. It is not conceivable that a small amount applied from outside would tip the balance and elicit contraction. It is more likely that the ATP applied from

outside acts on the process of excitation and not on contraction. It is an intriguing problem, to what extent excitation and contraction are built up on similar materials and similar processes.

Interpretation of Physical Changes in Muscle

Recent improvements in the registration of heat production (143) and the theory of Szent-Györgyi have prompted Hill to reinvestigate the early heat production during an isotonic twitch. His results (144 to 147) reveal the nature of the first event in heat production, the heat of activation. Heat of activation appears during the mechanical lag period and starts off at a maximum rate after a very short latency. Heat of activation seems to be quite independent of the conditions of muscle contraction. The "heat of maintenance" during a tetanus is regarded as the summation of the heat of activations associated with the elementary actions composing the tetanus.

The heat produced during a twitch is composed of the heat of activation and the heat of shortening. The latter is proportional to the shortening and primarily independent of the work done. Its value is of the order of 350 to 400 gm.cm. per cm. shortening per sq.cm. cross section of muscle. No heat is produced during relaxation if the load lifted by the muscle is detached before relaxation begins. If the load is left on, the equivalent of its potential energy is released during relaxation, but no more.

One of the most important conclusions of Hill is the revocation of his former statement about the sum of energy produced by muscle. The isotonic twitch being a more suitable and simpler subject of analysis, it is possible to study the heat production when the muscle is lifting various loads. It turns out that the work done appears always additional to the constant heat of activation and the corresponding heat of shortening. This is in direct contradiction to former ideas based on the study of the isometric tetanus.

Hill's myothermic measurements, while adding much to our knowledge, do again widen the gap between the interpretations given to physical and chemical studies. Hill himself, on the ground of the above evidence, objects to assuming any endothermic (or for that matter any exothermic) process to occur during relaxation. Hill even goes further, and points out (148) that no chemical measurements have ever proved the breakdown of ATP during contraction. Chemical determinations are obviously not suited for the study of these very fast changes, as they are limited by the need of fixing the tissue prior to determinations. Although the calculation of the adenosinetriphosphatase activity of muscle led Mommaerts (123) to a similar conclusion, these objections may be answered when the real rate of renewal of ATP inside the muscle fibre becomes known.

Millikan (149) correctly stated in 1942

> . . . the limits prescribed by the thermal data (in so far as they are adequately controlled) are inexorable, and . . . no theoretical mechanism can survive which violates them.

Yet it is obvious that Hill's standpoint is not undisputable: his arguments

are not valid against the participation of ATP in muscle contraction; they merely exclude the exothermic breakdown, a simple ATP hydrolysis during contraction.

It has been established that the rate of total energy liberation during an isotonic twitch, minus the heat of activation, is a linear function of the load. Hill refers to this relation as "simple and mysterious." It appears to the reviewer that this relation has been to some extent already cleared up by the investigations of Ernst (150, 151, 152).

The study of volume contraction by Ernst has shown that this event takes place not only in contracting muscle, but also in resting—not excited—muscle, when stretch is applied. In an isotonic tetanus, volume contraction was recorded and, during the tetanus, a sudden additional tension was applied. Again, volume contraction had set in, which was superimposed on that already existing due to the tetanus.

There are caoutchouc samples which show, when stretched, the following changes: volume contraction, increase of double refraction, sharpening of x-ray diagram, decrease of light transmission, etc. It is generally accepted that in the case of caoutchouc, the above phenomena are due to a crystallisation process, caused by the stress. Ernst points out that the same changes occur in stretched muscle, and advances the hypothesis that these too can be explained by assuming that a crystallisation process occurs in stretched muscle. Stretch causes crystallisation and this in turn leads to an increase of the resistance of muscle against further stretching. Ernst defines this phenomenon as the automatic part of muscle contraction. It appears to the reviewer that such an explanation would explain the relation between rate of energy liberation and load.

Hill (153) suggested that volume contraction is due to the internal pressure developing during contraction, because the fibres of the gastrocnemius are not running parallel. Ernst refutes Hill's objections (150) by showing that frog sartorius and semimembranosus muscles show the same volume contraction as gastrocnemii.

Finally, mention has to be made of some speculations on muscle. Riseman & Kirkwood (154) have revived the older theory of K. H. Meyer. They assume phosphorylation of myosin side chains during the splitting of ATP. They calculate the necessary number of changes in the charge of the myosin which would be needed to produce an elasticity change large enough to explain a coiling up of the myosin molecule chain.

Fleckenstein (155 to 157) developed a new osmotic theory of muscle contraction. Starting from the experimental result of Verzár & Somogyi (158), from the correlation between the loss of potassium and the work done, he calculates that the ionic change corresponds roughly to the same amount of energy. He therefore suggests that muscle is "charged" by the accumulation of potassium ions and contraction is accompanied by the exchange of the necessary amount of the accumulated potassium against sodium ions. A number of known facts can be convincingly explained by this theory, which undoubtedly describes one side of the whole story. But adrenalectomised

cats release much less potassium while performing the same work as normal animals (159). It is therefore questionable if the release of potassium is the first reaction which is energetically connected with muscle contraction.

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THE BIOCHEMISTRY OF NEOPLASTIC TISSUE[1]

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The ever-increasing number of publications in the field of cancer and tissue growth by investigators from every conceivable technical aspect is evidence of the effort that is being brought to bear upon this important problem. The literature has become so extensive that in a review of this type space limitations restrict the material to be covered. For this reason, very little will be said about the following categories: neoplasia in cold blooded vertebrates, just reviewed (1, 2); synthetic aspects of carcinogenesis, just reviewed (3, 4); morphology, pathology, immunology and viruses; and chemotherapy, recently reviewed (5). In general, the following topics will be considered: tumor constituents and metabolism, carcinogenesis, and possible diagnostic biochemical tools. In this paper an attempt has been made to review the literature which is of prime interest to the biochemist.

Tumor Constituents and Metabolism

Amino acids.—The amino acid composition of several normal tissues and of the tumors derived from them has been determined. With microbiological methods Roberts *et al.* (6) have compared the content of 12 amino acids (lysine, isoleucine, leucine, methionine, valine, phenylalanine, threonine, histidine, glutamic acid, cystine, arginine, and tryptophane) in normal and methylcholanthrene-treated mouse epidermis with that of a transplanted squamous cell carcinoma, originally derived from mouse epidermis by the topical application of the same carcinogen. The sum of the amino acids showed highly significant increases over the normal in the hyperplastic epidermis and in the carcinoma, whether expressed on a dry weight or on amino acid nitrogen bases per 100 mg. tissue. Benzene, the solvent for the carcinogen, produced changes in the distribution of amino acids in the epidermis which was different from that produced by the carcinogen in benzene in that the cystine content was higher and the methionine content lower than that of hyperplastic epidermis. However, the distribution of the amino acids in the carcinoma was quite similar to that of the precancerous epidermis and, therefore, showed no distinct pattern characteristically different from nonmalignant tissue. Roberts & Tishkoff (7) demonstrated by paper partition chromatography that hyperplastic epidermis and epidermis of new born mice, both of which are quite similar with respect to histological appearance and rate of growth, have larger amounts of free amino acids than the normal epidermis of mice. However, the carcinoma showed an overall

[1] This review covers the period from approximately November, 1948 to November, 1949.

decrease in content of free amino acids. A consistent pattern of free amino acid distribution was found in some malignant tissues, while most normal tissues differed from each other in this respect (8).

The amino acid analysis of a fibrosarcoma and its normal homologous connective tissue for 12 amino acids [aspartic acid and glycine, instead of tryptophane and cystine, the rest being the same as assayed by Roberts *et al.* (6)] was carried out by microbiological assays by Dunn *et al.* (9). Approximate values for cellular proteins were obtained since corrections were made for ash, fat, collagen, and elastin. The tumor contained a much higher content of arginine and glycine, an increase in threonine and lower levels of histidine and methionine, as compared to the tissue of origin.

Sauberlich *et al.* have carried out analyses for 18 amino acids in a rat hepatoma induced by azo dyes, a sarcoma induced by methylcholanthrene and the Flexner-Jobling carcinoma and found no significant difference between these tumors and normal muscle and liver. However, a spontaneous mammary carcinoma of the rat contained an abnormally high amount of glycine and proline and a relatively low content of histidine and methionine. The divergence of these values in the mammary carcinoma from the other tumors was attributed to the connective tissue present (10). Hence, there appears to be no distinctive amino acid pattern for the types of tumors analyzed.

Zamecnik *et al.* have found that the rate of incorporation of C^{14}-carboxyl-labeled DL-alanine into surviving slices of *p*-dimethylaminoazobenzene-induced hepatomas was seven times that of slices of control livers and 2.5 times that of nonmalignant portions of the liver (11). This incorporation was accounted for in the alanine fraction of the proteins. There was also an increased uptake in the hepatoma slices of C^{14}-carboxyl-labeled glycine as compared with normal liver slices. These experiments demonstrated that hepatoma cells incorporated alanine into protein more rapidly than did the normal liver cells, probably via a greater rate of peptide bond synthesis.

The horny excresence of Shope papillomas contained histidine, lysine, and arginine in average molecular ratios of 1:2.3:2.5 as compared to 1:3.3:3.1 for the carcinomas derived from them (12).

When DL-methionine tagged with S^{35} was given by gastric intubation to mice bearing spontaneous and transplanted tumors, a high initial activity was found in the tumors, liver, kidney, and intestinal wall (13).

In spite of numerous papers to the contrary (14), Kogl and his collaborators experimentally support their claims for the occurrence of D-glutamic acid in tumor tissue (15, 16).

The desoxyribonucleoproteins from necrotic and viable portions of an atypical epithelioma showed no qualitative difference in amino acids when measured by paper partition chromatography (17). The same amount of this nucleoprotein was found in normal and hepatoma liver cells by microphotometric estimation (18). A difference in the viscosity of the desoxyribonucleic acid complex of thymoma and thymus and a lack of a chromo-

somin-like protein in the latter have indicated that this complex from the thymoma is not the same as that of the tissue of origin (19).

A striking increase in the accumulation of radioactive iodine in the skin of rats bearing a fibrosarcoma or an adenocarcinoma, and in the skin of mice bearing a lymphoma, hepatoma, or mammary carcinoma after receiving tagged thyroglobulin was reported by Scott *et al.* (20). Curtis-Dunning rats bearing a transplanted pancreatic carcinoma did not show the skin increase which was attributed to the type of tumor, since there was an increased accumulation of I^{131} in other tissues. The tumors did not take up much thyroglobulin. The authors suggest that the higher skin uptake of the globulin-iodine fractions in the presence of a tumor is a reflection of whole body dysfunction. N-iodoacetyl derivatives of tryptophane, leucine, phenylalanine, and iodoacetamide containing I^{131} inhibited significantly the growth of sarcoma 37 in a manner unrelated to their toxicity. Radioactivity from these substances has been found in significant amounts in blood, tumor, and liver, the activity in the tumor being greater than that of liver (21).

Glycolysis.—The investigations of Warburg (22) established that the rate of glycolysis is high in tumors, but only recently have further aspects of physiological significance of this phenomenon been explored. Le Page has found most of the components of the Embden-Meyerhof phosphorylative glycolysis system to be present in primary and transplanted tumors of the rat and mouse (23, 24). There was a high rate of glycolysis in homogenates of these tumors when supplemented with all of the known accessory factors. It was also shown that homogenates of these tumors formed phosphoglyceric acid and lactic acid at about the same rate via a coupled oxido-reduction (25).

Conditions were devised for glycolysis in homogenates of the Flexner-Jobling rat carcinoma which permitted glycolysis of glucose, esterification of inorganic phosphate, and the maintenance of phosphate-bond energy (adenosinetriphosphate) (26). Under the same conditions the desoxyribonucleic acid and ribonucleic acid were maintained when the homogenates of the tumor were provided with adenosinetriphosphate by means of an active anerobic glycolysis (27). When the latter was not maintained, there was a decrease in the level of the nucleic acids. The glycolytic enzymes of the Flexner-Jobling tumor, as measured aerobically under optimal conditions, were largely in the nonsedimentable or "soluble" fraction (28).

It has been suggested that glycolysis can serve as a source of energy for the incorporation of phosphate into nucleic acid and phosphoprotein (29).

Meyerhof & Wilson have also shown that homogenates and centrifuged extracts of a rat sarcoma maintained a steady and rapid rate of glycolysis of free sugar in the absence of large amounts of phosphate donors, provided sufficient hexokinase and adenosinetriphosphatase were present (30). This ratio was made possible by the inhibition of adenosinetriphosphatase by toluene, or octyl or decyl alcohol, or by the addition of yeast hexokinase. The adenosinetriphosphatase activity of normal tissues was not appreciably

affected by octyl alcohol. The above conditions held true for both rat and mouse sarcomas and carcinomas (31). Octyl and decyl alcohols and 0.01 *M* sodium azide inhibited the adenosinetriphosphatase activity in different types of tumors of both animals. Homogenates of mammary carcinoma of C_3H mice glycolyzed fructose and glucose at the same rate, while the other sarcomas and carcinomas studied glycolyzed fructose at only a fraction of the rate of glucose. The adenosinetriphosphatase activity of tumor homogenates was inhibited not only by higher alcohols, but also by digitonin, while that of chick embryo was inhibited by 0.02 *M* sodium azide, but not by narcotics (32).

Studies on the oxidation of oxaloacetate by tumor tissue has been continued by Potter and co-workers (33). Optimal conditions for the oxidation of oxaloacetate in tissue homogenates have been devised. An accumulation of citrate was found with liver and kidney, but not with brain, heart, and tumors. In the tumors studied, the oxidation of oxaloacetate was negligible, while in brain and heart there was considerable utilization of the substrate with fortified homogenates. Even in the presence of an active phosphorylative glycolysis fortified homogenates of the Flexner-Jobling tumor were not able to oxidize oxaloacetate (34).

The citric acid content of Walker 256 rat tumor was higher in the nongrowing portion than in the viable periphery, while aconitase activity was present in the latter but not in the former (35).

Enzyme activities.—It has been shown that the rate of hydrolysis of 2,4-diketo acids by acylpyruvase in homogenates of mouse and rat hepatomas was considerably lower than that of the livers of both animals and was much lower for the rat hepatoma than for that of the mouse. Similar observations were made on the rates of hydrolysis of a series of other diketo acids (36). The dehydropeptidase activity with alanyldehydroalanine and glycyldehydroalanine as substrates was about the same for normal and tumorous tissues. On the other hand, the rate of hydrolysis of glycyldehydrophenylalanine and for N-methylglycyldehydroalanine was small or neglible in tumors, but high in kidney and liver (37). The carbonic anhydrase activity of livers of rats made precancerous by dietary *p*-dimethylaminoazobenzene was about the same as that of normal liver, but the activity of this enzyme fell to about 50 per cent of the normal in the hepatomas (38). The growth of the tumor had no apparent effect upon the carbonic anhydrase activity of the host tissues. The decrease in the liver catalase of rats bearing the Jensen sarcoma was found to be independent of the protein content (18 to 45 per cent) of the diet (39). A thermostable, nonheat-coagulable, water-soluble and alcohol-precipitable protein-like material, isolated from both sarcomas and carcinomas, has the property of lowering the liver catalase of mice (40). This material was not found in normal tissues.

The cathepsin-activating ability of ultrafiltrates from hepatomas was less than that of normal rat livers, while the glutathione content of the latter tissue was higher than that of the former. Since glutathione appears to be a major cathepsin-activating agent, the authors believe that the in-

creased growth rate of the hepatomas may be related to the lower concentration of this tripeptide and a decreased functioning of the protein-degrading mechanisms (41).

Carcinomas of the cervix and vagina contained a high activity of β-glucuronidase (42), while the activity of this enzyme in adenocarcinomas of the uterus was about the same as that from the endometrium with benign uterine bleeding (43). Assays of β-glucuronidase in vaginal fluid showed a high activity of untreated cervical cancer, but there were 18 per cent false positives in benign conditions of this organ. Following irradiation, the activity of this enzyme decreased in the cervix and in the vaginal fluid.

No significant differences in total dehydrogenase activity of mouse liver, kidney, muscle, brain, and spleen were found in comparison with the same organs in mice bearing transplanted tumors of various types (44). Furthermore, the activities of malic and lactic dehydrogenases in the same organs of mice with and without certain transplanted tumors were the same (45). The values for these enzymes in a transplantable adenocarcinoma and a sarcoma were lower than those of the normal tissues, with the exception of malic dehydrogenase activity of spleen of both normal and tumor-bearing mice which was practically zero.

The granules (mitochondria) isolated by centrifugation from the Algire S91A, Cloudman S91, and Harding-Passey mouse melanomas contained cytochrome oxidase and succinic dehydrogenase activities and cytochrome-*c*. The amelanotic S91A granules did not have tyrosinase or dopa-oxidase activity, in contrast to the granules of the Harding-Passey melanoma which possessed dopa-oxidase, but no appreciable tyrosinase activity (46).

Melanized granules of the Cloudman S91 and Harding-Passey melanomas have been separated from other cellular constituents by adsorption on Celite (47). The succinoxidase and dopa-oxidase activities of these homogenous granules were sixfold and tenfold, respectively, over that of the initial extract prepared by centrifugation. Nearly all of the protein of the transplanted melanoma S91 is a water-soluble pseudo-globulin to which practically all the tumor melanin is attached (48). The dried melanin to which this protein is associated contained nitrogen, 15 per cent; sulphur, 1.6 per cent; cystine-cysteine, 2.6 per cent; methionine, 2.0 per cent; tryptophane, 1 per cent, and no phosphorus. By chemical means nearly pure melanin was obtained from the melanin-containing protein of the S91 melanoma and from pigmented human melanomas. This pigment from both sources contained tyrosinase and dopa-oxidase activities (both cyanide sensitive) and a potent system (cyanide insensitive) which oxidized *p*-phenylenediamine. Nonpigmented melanomas did not have these enzyme systems. The tyrosine present in the S91 melanoma and in some other organs of mice after the intravenous injection of DL-tyrosine labeled with C^{14} in the beta position contained a high specific activity (49).

Hyaluronidase, determined by the mucin clot-prevention test, was found in only two of 17 human tumors and in two of 19 varieties of spontaneous and transplanted animal tumors (50).

The arginase activity of transplantable squamous cell carcinomas was 11 to 33 times that of normal mouse epidermis on a total nitrogen basis. The value for hyperplastic epidermis was about the same as that of normal, except for the epidermis which was painted three times with methylcholanthrene, in which the arginase activity was about three times that of the normal (51). Six and 11 paintings of benzene alone produced a twofold increase in the activity of this enzyme. The urea content of hyperplastic epidermis decreased from about 50 per cent and the level of this substance in the carcinoma to about one-third that of the normal epidermis. No change occurred in the ammonia content during carcinogenesis, but the total nitrogen and trichloroacetic acid-soluble nitrogen decreased in the carcinoma.

Bodansky has shown that human osteogenic sarcoma phosphatase resembled normal rat bone phosphatase with respect to the extent of inhibition by glycine, alanine, histidine, and glutamic acid, and the decrease of an activating effect of magnesium or even its reversal in the presence of inhibiting concentrations of amino acids or cyanide (52). The phosphatases of these tissues were also similar in the shift of the pH optimum to 10.5 in the presence of cobalt and amino acid and in the requirement of both cobalt and magnesium for the counteraction of the inhibition by amino acids.

No significant differences were found in the adenosine-triphosphatase activities of various malignant tumors and normal tissues of mice and rats (53). Most of the activity of this enzyme in normal and cancerous tissues was in the water-soluble fraction (54). The adenosinetriphosphatase activity of the water-soluble globulin type of structural proteins of dibenzanthracene-induced tumors or of Brown-Pierce carcinomas was not changed significantly after repeated reprecipitations from solution (55).

Diethylstilbestrol has been found to inhibit competitively the oxygen consumption via the succinoxidase system of the mitochondrial elements of the S91 and Harding-Passey melanomas. This inhibition was reduced or eliminated by the simultaneous addition of progesterone or testosterone (56). Inhibition by diethylstilbestrol also occurred in the same particulates of C3HBA breast carcinoma and sarcoma 37. Alpha and beta peltatin caused a reduction in the cytochrome oxidase activity of homogenates of sarcoma 37 to 40 per cent of the normal untreated tumors within 8 hr. after a 20 μg. injection. This dose did not appreciably affect the activity of this enzyme in the liver, spleen, and kidney. Even a lethal dose failed to affect the cytochrome oxidase activity of these organs (57).

Carcinogenesis

By hydrocarbons.—The solubilization of carcinogenic hydrocarbons by sodium oleate and cholate and by Triton NE[3] to give clear, homogenous, and stable solutions is possible (58). Carcinogens applied in such solutions locate in the skin of mice in the same manner as when dissolved in the usual organic solvents. Carbowax and dioxane as solvents for hydrocarbons are also

[3] Triton NE, a non-ionogenic association colloid, an alkyl polyether alcohol.

suitable for skin carcinogenesis in mice, and the response of the skin is dependent upon the solvent used (59). Human sebum did not have an anticarcinogenic effect upon epidermal carcinogenesis in mice when mixed with methylcholanthrene, whereas lanolin or sheep sebum had a pronounced influence in retarding carcinogenesis (60, 61).

Hieger reported that the injection of the unsaponifiable fractions of human and animal tissues resulted in the induction of 63 tumors in some 2,000 mice at the locus of the material. The carcinogenic factor may be cholesterol, or cholesterol combined with a small amount of a frequently occurring, unknown co-carcinogen (62).

The liver of rats maintained on a 20 per cent protein diet showed an increased ascorbic acid content after the intraperitoneal injection of 1,2,5,6-dibenzanthracene. This increase arose before any growth inhibition or decrease in liver succinoxidase was noted. The extent of the ascorbic acid rise in the liver of rats maintained on 5, 10, and 20 per cent protein diets was roughly proportional to the amount of growth inhibition (63). The direct chemical action of 3,4-benzpyrene with sulfhydryl groups of cysteine hydrochloride, thiomalic acid, thioglycollic acid and 2-mercaptoethanol suggested a parallelism with the metabolism of this hydrocarbon after introduction into the animal body. The fluorescence spectra and the physical properties of the above compounds were quite similar to benzpyrene metabolites (64).

The distribution and metabolism of 20-methylcholanthrene-11-C^{14} followed the same pattern as for dibenzanthracene (65).

Benzpyrene, dissolved in tricaprylin, remained at the site of injection in mice in progressively diminishing amounts up to six months, and during the latent period a metabolite was found by spectrographic analysis (66). Domestic soot has been found to have a benzpyrene content of 300 mg. per kg. which was identified by fluorescence spectroscopy after purification by chromatography (67).

Ultraviolet fluorescence microscopy has been used again for the localization of carcinogenic hydrocarbons in the skin of mice and rabbits (68, 69).

Some of the compounds formed by pyrolysis of cholesterol have been separated and identified by Faulk *et al.* and the majority of them are intermediates in the laboratory synthesis of methylcholanthrene from cholesterol (70). The possible rôle of these intermediates in carcinogenesis will be eagerly awaited, since the theory of endogenous carcinogen formation is one long held by some investigators.

Recently evidence was presented for the existence of a qualitative chemical change in carcinogenesis (71). This evidence was uncovered by a difference in the type of polarographic waves and the half-wave potentials of the reducible material extractable by alcohol and ether from normal and methylcholanthrene-treated mouse epidermis and from a transplantable squamous cell carcinoma originally derived from the latter tissue. The reducible material from normal and hyperplastic epidermis had a single polarographic wave in buffered solutions at pH 4.4, 5.8, and 7.1 and a double wave at pH 6.3 to 6.7, while that from the carcinoma had only a single wave at these pH

values. Moreover, the half-wave potentials of the material from epidermis was 200 mv. more negative at pH 7.1 than that of carcinoma, and about 100 mv. more negative at the lower pH values (72). The diffusion current of the single wave of the material from the carcinoma and of the single wave at the lower pH values and of double wave of epidermis at pH 6.3 to 6.7 was proportional to the concentration of the reducible material. The polarographic characteristics of the substance in the epidermis are changed to that of the carcinoma as soon as the carcinomas appear after induction by methylcholanthrene and are the same in transplantable squamous cell carcinomas of different degrees of anaplasia. Normal human epidermis contains a substance indistinguishable polarographically from that of the mouse and it also undergoes alteration in squamous cell carcinomas of man.

A second paper describing another qualitative chemical change in carcinogenesis was reported by Kidder *et al.* (73). This discovery was based upon the fact that the protozoan *Tetrahymena geleii* cannot synthesize guanine, but requires it in its diet (74), while the mammal can synthesize guanine from adenine (75). Then the substituted purine 5-amino-7-hydroxy-1H-v-triazolo-[d]pyrimidine (triazologuanine) was found to be a powerful growth inhibitor for *T. geleii* which could be completely reversed by guanine (76). When mice bearing transplanted tumors (adenocarcinoma Eo771, spontaneous mammary carcinoma in C_3H mice, and lymphoid leukemia) were injected with triazologuanine, a nontoxic substance, the growth of the tumors was stopped completely, but was renewed upon cessation of treatment. These data suggest that these tumor cells have a metabolism similar to that of *T. geleii* in contrast to that of normal tissue cells.

A tumor antigen of lipid character, capable of inciting the formation of specific antibodies in the rabbit, has been isolated in a high state of purity from a human mammary tumor (77).

Diet and carcinogenesis.—The incidence and average time of appearance of spontaneous mammary carcinoma were both accelerated by increasing the fat content of the diet which was fed equicalorically at slightly below *ad libitum* levels (78). The rate of epithelial tumor formation by benzpyrene in mice was likewise increased by dietary fat, and the stimulating effect was probably associated with the ability of the fat to augment the net energy value of the diet (79). On the other hand, diethylstilbestrol-induced mammary cancers in rats were not appreciably affected by variations in the fat and carbohydrate content of the diet (80). The incidence of mammary carcinomas and of skin tumors and sarcomas by carcinogenic hydrocarbons was not significantly changed when the dietary protein ranged from 9 to 45 per cent. Only the incidence of spontaneous hepatomas was significantly lower in mice on the low protein diet (81). Caloric restriction may reduce the extent of mammary carcinoma formation by a suppression of the endogenous estrogen production, since ovariectomized C_3H mice supplemented with 0.5 μg. of diethylstilbestrol per day showed a 100 per cent incidence of mammary tumors whether fed *ad libitum* on a diet restricted in caloric intake (82).

The starvation of mice for 36 hr. reduced the mitotic activity of the epi-

dermis of the ear to a very low value, while restriction of the diet to 50 per cent of that consumed by controls reduced the mitotic activity by 85 per cent. The prevention of tumor formation by dietary restriction may be explained by these studies (83). However, the induction of skin papillomata by a single application of benzpyrene or methylcholanthrene to the interscapular region followed by repeated doses of croton oil was independent of the number of epidermal mitoses during the time of action of the carcinogen (84).

Feeding diets of varying vitamin content had no effect upon benzypyrene-induced tumors in mice (85). A choline-deficiency of long duration in chickens resulted in the appearance of many types of tumors (86) such as was first observed in rats under a similar dietary regime (87). Alimentary fats had only a slight influence on the formation of liver tumors in rats on a choline deficient diet (88).

The growth of the Walker carcinoma 256 increases the caloric requirements of the host since sexually mature rats bearing this tumor lost significantly more total lipid than did pair-fed controls of the same age, weight, and sex (89). The carcass lipids of rats bearing this same tumor varied inversely with the size of the tumor (90). In most cases the blood lipids, chiefly fatty acids, were increased several times above the normal. The adrenals of animals bearing tumors weighed as much as three times the normal adrenal and the average total steroid content decreased to about one-third of the latter.

By azo compounds.—The biochemistry of the transformation of liver to hepatoma by azo dyes has received considerable attention during the past year. Although the incidence of hepatic tumors induced by *p*-dimethylaminoazobenzene, o′-methyl-*p*-dimethylaminoazobenzene and *m*′-methyl-*p*-dimethylaminoazobenzene was less on a diet containing 12 to 24 per cent casein plus methionine than on a 12 per cent casein diet, the effect was not marked. Both casein and methionine were effective in improving the hepatic content of riboflavin during the feeding of the azo dyes (91). Five to 20 per cent of cottonseed oil in the diet had no influence upon the development of hepatic tumors by *p*-dimethylaminoazobenzene, whereas diets containing 20 per cent corn oil resulted in a more rapid development of tumors than with diets containing 5 and 10 per cent of this oil. Retardation of hepatic carcinogenesis was marked with 100 mg. of riboflavin per kg. of diet containing 10 per cent casein (92).

A fairly high incidence of tumors results from the intermittent application of carcinogens to the skin (93, 94, 95) and a similar effect has been shown to occur in the induction of hepatomas in the rat after feeding *m*′-methyl-*p*-dimethylaminoazobenzene. Nicotinamide, 0.29 per cent, corn oil, 20 per cent, and restriction of the diet to 63 per cent of the calories consumed by the controls during the intermittent period on the dye-free regime increased the incidence of tumors, while choline and methionine had no such effect (96). The incidence of tumors of the urinary bladder of rats induced by the ingestion of azotoluene and its split products and by aniline was influenced by the riboflavin and protein contents of the diet (97).

Kensler has shown that the ability of liver slices to destroy N,N-dimethyl-*p*-aminoazobenzene is increased when the riboflavin content rises. Thiamine, choline, biotin, or adenine had no effect upon the liver riboflavin content and hence no influence on the destruction of the carcinogen (98). The metabolism of compounds related to N,N-dimethyl-*p*-aminoazobenzene under similar conditions has been summarized (99).

Cook *et al.* have observed that incorporation into the diet of the potent carcinogen m′-methyl-*p*-dimethylaminoazobenzene increased the γ-globulin content and decreased the albumin content of rat serum while *p*-dimethylaminoazobenzene, a less potent carcinogen, had less effect upon these proteins (100). The noncarcinogenic dye, *p*-aminoazobenzene, or acetylaminofluorene after six weeks of feeding had no such influence. These azo dyes did not alter the mobilities of the serum proteins.

The serum proteins of rats fed *p*-dimethylaminoazobenzene increased markedly at the time of occurrence of tumors, the elevation being independent of the size of the latter. There was a relative and absolute increase in the globulins, mainly beta and gamma. The serum albumin was unchanged, but generally the albumin electrophoretic peak was not resolved into two compounds as in normal rat sera. Changes in the serum proteins may be associated with an immunological reaction (101).

The nature of the chemical alterations in the liver resulting from the ingestion of azo dyes has been studied by several investigators. Griffin and co-workers have found that *m′*-methyl-*p*-dimethylaminoazobenzene induced a gradual increase in the desoxyribonucleoproteins of normal rat liver of 266 mg. per cent to a value of 506 mg. per cent after eight weeks feeding of the dye to a value of 756 mg. per cent in the hepatoma (102). On the other hand, the riboflavin content decreased, the globulin level increased, while the nitrogen, phosphorus, ribonucleoproteins remained fairly constant during the precancerous stage.

Miller, Miller and their co-workers have continued their investigations on the chemistry of hepatic carcinogenesis. Homogenates and cellular fractions from rat livers fed 4-dimethylaminoazobenzene for four to five months were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dyes (103, 104). In the preneoplastic stage the levels of protein and desoxyribonucleic acid were increased in the nuclear fraction, the contents of protein, riboflavin, and pentosenucleic acid were decreased in the large granules, the latter was also decreased in the small granule fraction, but increased in the supernatant and in the nuclear fraction. The induced tumors contained higher levels of protein and desoxypentosenucleic acid in the nuclear fraction than did this same fraction of livers from rats fed the dye, but the large granule fraction of the tumors had about one-half the amount of protein, pentosenucleic acid, and riboflavin as the precancerous livers. The tumor fractions did not contain any detectable amounts of protein-bound dye. The highly active carcinogen, 3′-methyl-*p*-dimethylaminoazobenzene, produced many of the same changes but of greater magnitude in the composition of livers as were found for 4-dimethylaminoazobenzene

(105). Some of the changes induced by the former simulated those of hepatic tumors. On the other hand, the weakly carcinogenic dye, 4′-methyl-*p*-dimethylaminoazobenzene, and the noncarcinogenic dye, 4-aminoazobenzene produced little or no change in the composition of the liver. Protein-bound aminoazo dye was found in each fraction of the liver cell, but there was no apparent relationship between carcinogenicity and binding.

The same investigators have carried out detailed studies on the binding of azo dyes by liver proteins aided by the development of micromethods for the quantitative determinations of many of the reduction products of these carcinogens (106). Reduction of the polar bound dyes isolated after the ingestion of 4-dimethylaminoazobenzene and of substituted methyl derivatives of this compound indicated that the dyes are bound to protein either through the $-N(CH_3)_2$ group or to the ring bearing this group. The levels of bound dye of 4-dimethylaminoazobenzene and its 2-, 2′-, 3′- and 4′-methyl derivatives, and of 3-methyl-4-monomethylaminoazobenzene during a period of one to 21 weeks of feeding showed an inverse relationship between carcinogenic activity and the time for maximum binding of the dye for the methyl derivatives of the prime ring. This relationship was further demonstrated by comparing the more rapid binding of 4-dimethylaminoazobenzene at a higher dietary level with a quicker response to tumor formation. The authors suggest that the initial tumor cells of the liver are a result of an alteration of one or more of the specific synthetic mechanisms of the liver cell so that the latter are unable to keep pace with the continual demands of normal function and removal of protein by the carcinogen. The solution of the mechanism of azo-dye binding and its physiological rôle should add much to our knowledge of hepatic carcinogenesis.

Steric factors are probably the dominating considerations which determine the carcinogenic activity of ring fluoro- and trifluoromethyl derivatives of 4-dimethylaminoazobenzene (107).

The livers of rats poisoned with a single large dose of *p*-dimethylaminoazobenzene failed to store glycogen even after the ingestion and absorption of large amounts of carbohydrate (108). Hepatoma cells produced by this carcinogen absorbed less water and underwent more rapid disintegration than did normal liver cells (109).

Paper partition chromatography has been applied to the study of azo dyes and their metabolites (110). The use of fortified rat liver homogenates in the study of the metabolism of 4-dimethylaminoazobenzene led to the isolation of 4-monomethylaminoazobenzene, 4-aminoazobenzene and a new metabolite, 4′-hydroxy-4-dimethylaminoazobenzene (111). The metabolites did not account for all the dye metabolized. A synthesis of *p*-dimethylaminoazobenzene containing N^{15} as a tracer element in each of the three possible positions for metabolic studies is available (112).

The latent period of the auto-oxidation of linoleic acid was increased by *p*-dimethylaminoazobenzene and *p*-monomethylaminoazobenzene, the former being more effective than the latter. During auto-oxidation of the azo dye mixtures demethylation of the two dyes occurred and 85 per cent of

the dimethyl derivative appeared as the monomethyl derivative. Demethylation was prevented by the addition of tocopherol (113).

Although the azo dyes are usually considered carcinogenic for the liver only, lymphoblastic lymphosarcomas have been induced in the spleen of rats by intrasplenic implants of *p*-dimethylaminoazobenzene (114). Three pancreatic tumors were also obtained in 3 of 17 rats fed this same compound in a diet not conducive to the formation of hepatic tumors in two of the animals (115).

By acetylaminofluorene.—Acetylaminofluorene, but not fluorene, produced not only tumors of the skin of rats when applied topically, but also in some internal organs (116). Alterations in the structure of 2-acetylaminofluorene by replacing the methylene bridge with a sulfur atom as in 3-acetylaminodibenzothiophene did not alter its carcinogenicity for the mammary gland or ear duct, but substitution of a sulfone grouping as in 3-acetylaminodibenzothiophene-5-oxide greatly lowered the carcinogenic activity for these two tissues. Insertion of an oxygen bridge as in 3-acetylaminodibenzofuran partially lessened the carcinogenic activity. None of these analogues, unlike 2-acetylaminofluorene, produced liver tumors (117, 118). The fact that 4-dimethylaminobiphenyl, a derivative in which the methylene group is absent, is a powerful carcinogen, is not in harmony with Pinck's theory of carcinogenesis (119). For distribution and metabolism studies a synthesis of 2-acetylamino-9-C^{14}-fluorene is available (120).

Carcinogenesis of the liver produced by feeding acetylaminofluorene in the diet caused a decrease in the desoxyribonucleoprotein content of this organ in contrast to an increase produced by azo dyes. The concentration of ribonucleoprotein, riboflavin, nitrogen, and phosphorus also fell during carcinogenesis. Liver tumors induced by this agent had lower amounts of desoxyribonucleoprotein, nitrogen, and phosphorus than did azo dye-induced hepatomas (121). The difference in the chemical composition of liver tumors produced by azo dyes and acetylaminofluorene may be partially explained on the type of growth induced, hepatoma, or bile duct carcinoma, by the latter carcinogen.

N-acetyl-2-aminofluorene is widely distributed throughout the body of rats over a 16-hr. period following a single feeding. Three hours after administration the greatest concentration was found in the plasma, liver, kidney, muscle, and brain. At dosages of 3.1 mg. per 100 gm., the carcinogen persisted in the tissues for as long as 6 hr. in muscle to 16 hr. in liver and kidney (122). A high dietary concentration of this carcinogen will induce a lowering of plasma albumin (123).

Viruses

The isolation and some properties of the virus of mouse mammary carcinoma have been reported recently by Graff *et al.* (124, 125). The highly purified virus particles produced mammary carcinoma in susceptible mice. The virus absorbed maximally at 260 mμ. which is characteristic of nucleic acid, and in the ultracentrifuge the material separated into two boundaries, one sharp with sedimentation constant of 900 S. (Svedberg) units, and another

diffuse with an S constant of 500 to 700. Electrophoretically the virus migrated in two components, both of which were active but no separation of them was possible. The virus particles could not be obtained from the milk of C57 mice which exhibit practically no spontaneous mammary carcinomas.

Recent experiments of Gye and Mann have raised the question of viral etiology of other mammalian tumors (126 to 130). The active form of the "Bittner" virus was obtained by freezing mammary tumors at −79°C. The refrigerated material had selectivity for mammary tissue only, while viable tumor cells, as is well known, will grow in many places in the host. An active form of the "Bittner" virus in the dried state was obtained by a procedure in which the tissue was minced in a special device (131), diluted with 5 to 10 per cent glucose or glucose and glycerol (132), and stored at −79°C. for varying periods of time. Then the suspension of tumor cells was thawed, and dried rapidly with the Craigie drying apparatus (133). This dried material produced mammary tumors in both male and female mice upon injection into breast tissue. A sarcoma, C48, originally induced by methylcholanthrene, and two other transplantable sarcomas of spontaneous origin yielded active dry material by the same procedure (134). From these investigations the authors concluded that "the apparent gap between filterable and nonfilterable tumors is fictitious; the difference between chicken tumors and mammalian tumors is quantitative and not qualitative." Embryonic cells of mice exposed to a temperature of −79°C. for a few hours lost their transplantability, although untreated coarse and fine mince of embryos lived and grew when implanted under the skin of the same inbred strain (135).

Some pertinent questions regarding the investigations of Gye and Mann have been raised (136). "Has the dead tumor been cultivated *in vitro?* Have the respiration and other metabolic processes of the frozen tissue been sufficiently studied? Can the new virus be filtered and centrifuged? What proof is there that the tumor tissue was perfectly dry?" It was concluded that: "The weakness of his (Gye's) position is that he is balancing one assumption, the mammalian virus on another assumption, the freeze-death of the cells."

Miscellaneous

Studies with 2,5-diphenyl-3-*p*-radioiodophenyl tetrazolium chloride have demonstrated that neoplastic tissues *in vivo* or *in vitro* were not able to reduce this compound more readily or extensively than most normal tissues (137).

The radioactivity found in a mammary spindle cell carcinoma in mice after the administration of trypan blue containing radioactive iodine was higher than that found in skeletal muscle and skin and lower than the activity of liver, spleen, or kidney (138). Radioactive sodium iodide, injected subcutaneously into rats with and without Walker tumor 256, revealed that the activity of the injected iodine was lower in the thyroid and other tissues of the tumor-bearing rats than in the controls. This difference may have been due to the lower concentration of radioactivity in the sera of the tumor-bearing rats (139).

Normal mouse epidermis has shown an active exchange of radiocalcium. In early methylcholanthrene-induced hyperplasia there was a loss in storage capacity for calcium. Epidermis in later hyperplasia and in a transplantable squamous cell carcinoma was completely unable to exchange this element. This characteristic of the carcinoma is believed to result from an alteration in the calcium-binding protein complex at the cell surface (140).

Possible Diagnostic Tools

During the past year considerable attention has been focused on the development of chemical tests for malignancy in man, and a résumé of them at the present time seems worthwhile. Alteration in serum proteins in cancer have been reviewed by Huggins (141) and an anomaly in the thermal coagulation of serum proteins has been observed by him and his co-workers (142). Eighty-five patients with clinically active cancer exhibited this abnormality, as did 16 of 95 patients with non-malignant diseases.

The use of the polarograph for the serological detection of cancer according to the procedure of Brdicka (143, 144) and others has been employed again. This method is particularly suited for the detection of prostatic cancer, and the repeated examination of the sera gave a valuable indication of the effectiveness of therapy (145). A perfect correlation between cancer and the polarographic diagnosis was found for 20 patients, but the results were falsely positive in 20 to 25 per cent of normal persons (146). Many others have shown that the polarographic method is not specific for cancer (147, 148).

Increased serum aldolase content of rats bearing sarcoma 37 and Walker carcinosarcoma 256 has been demonstrated by Sibley & Lehninger (149) in confirmation of the work of Warburg & Christian (150). Such factors as cachexia, anemia, infection, and pregnancy did not cause a decrease in the serum aldolase levels. The latter fell to normal levels upon extirpation of the tumor. On the other hand, only 20 per cent of 104 cases of human cancer had a significant rise in serum aldolase.

A significant increase in the inhibition of hyaluronidase by the serum from human cancer patients over that of normal persons was found (151). Also a thermolabile hyaluronidase inhibitor is increased in a major proportion of sera of cancer patients, but some of the latter were indistinguishable from the normal. Rheumatic fever and arthritis showed no deviation from normal (152).

A marked increase in the serum polysaccharide (nonglucosamine) occurred in patients with malignancies in 96 per cent of 105 patients, but many other pathological conditions caused elevations in this substance (153). A serological test for malignancy based upon the flocculation of cancer sera with the unsaponifiable fraction of human cancerous liver gave positive results in 80 to 98 per cent of cancer patients and in 20 to 25 per cent of other pathological conditions (154).

Gamma globulin in six of seven patients with multiple myeloma was

distinguishable by ultracentrifugal monodispersity and electrophoretic homogeneity from that of normal γ-globulin (155). Blood histamine is raised to very high levels in chronic myelocytic leukemia, is lowered in acute myelocytic leukemia and in acute exacerbations of the former, and is unchanged in lymphatic and monocytic leukemias as compared to normal (156).

The claims of Hirshfeld *et al.* (157) on a factor in the blood serum of cancer patients which inhibits the aerobic oxidation of tryosine by crude potato tyrosinase was not confirmed in a thorough investigation by Marx (158). The use of aniline dyes for the detection of malignancy in sera with a high degree of positive results has been claimed (159). The employment of a diphenylamine colorimetric reaction for malignancy has been suggested, although many other pathological conditions give colors of lower intensity than that given by cancer sera (160).

The excretion of 11-β-hydroxyetiocholanolone, a compound present in the urine of a significant number of patients with neoplastic disease, but rarely in the urine of normal individuals, was found in the urine of patients with gastric cancer by Dobriner *et al.* (161). A large increase in the excretion of 17-ketosteroids in the urine of nine of ten patients with adrenal cortical tumors was observed, and by chromatographic techniques marked differences were found in the amounts of eight individual 17-ketosteroids excreted by these patients as compared to normal (162). Androsterone and aetocholan-3-(α)-ol-17-one were increased in the urine of patients with prostatic hyperplasia and cancer as compared to normal males (163). Urinary cholesterol, which is normally associated with albuminuria or pyuria (164) is increased in about 10 per cent of cancer patients after corrections are made for the cholesterol associated with the aforementioned conditions (165).

There are at present no reliable diagnostic tests of a biochemical nature which are capable of detecting malignancy in its earliest stages.

It is apparent from the literature reviewed that many different approaches are being brought to bear upon this important problem. A wide variety of agents are capable of producing tumors of many diversified types and the mechanism by which certain classes of carcinogens induce new growths is being intensively pursued. On the other hand, there is the chemical similarity of tumors as expressed by Greenstein (166) in that "It seems probably that a cancerous tissue can be described by a chemical pattern which is largely similar to that of nearly all other cancerous tissues regardless of their etiology, histogenesis, or even species wherein found." Nevertheless the mechanism by which different carcinogens promote the origin of neoplastic growths is probably not the same, primarily because of the tissue specificity of some carcinogens and the nonspecificity of others. A better understanding of the intermediate stages is almost obligatory to ascertain the manner in which normal cells lose their properties of controlled growth and function by a particular carcinogen, in spite of the fact that most tumors have much in common biochemically.

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CHEMICAL COMPOSITION OF BLOOD PLASMA AND SERUM[1]

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Recent advances in analytical technique have produced many new data on the chemical composition of tissues and body fluids. Blood plasma, as the environment of the tissues, occupies a special place among these materials and details of its composition are therefore of general interest. No survey of the chemical composition of blood plasma seems to have been made recently, apart from reviews of the clinical aspects [Rourke, MacLachlan & Butler (1) and Sunderman & Boerner (1a)], and it was therefore thought useful to collect and sift the available information in this field. The subject is dealt with from the general biological standpoint, consideration of space precluding a discussion of the clinical aspects. The majority of the data discussed in the review refer to man, the most thoroughly studied species.

For brevity's sake, most of the data are arranged in Tables. Where more than one reference is given, the first is usually the paper from which the data are taken. Other references contain confirmatory results and/or values for other species.

In order to make the review comprehensive, an attempt has been made to include in the Tables data for all normal plasma constituents for which reliable quantitative information is available, but only more recent results are discussed in the text. In a few cases where no values for plasma or serum are available, data for whole blood are given. This is indicated in the first column of the Tables.

Inorganic Constituents

Numerous determinations of the main inorganic plasma constituents have been published in recent years. Some new ground has been covered in respect of various trace metals. As far as the major constituents are concerned the newer analyses have been essentially confirmatory, the main point of the new techniques being simplification and greater accuracy. Representative data on human plasma or serum are given in TABLE I.

The only items warranting comment are the values for the iodine and for nonprotein sulphur. In human plasma "protein-bound" iodine and "total" iodine appear to be virtually identical whilst in other species, e.g., chicken and notably dog, total iodine much exceeds the protein-bound iodine (12). There is evidence suggesting that the protein-bound iodine is identical with thyroxine [Harington (34), Taurog & Chaikoff (35)]. The figures given in the literature for the non-protein sulphur, except inorganic sulphate,

[1] This review covers the literature until the end of 1949.

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show some discrepancies. Sturm & Pothmann (29) reviewed data published by 18 different authors. The value for "neutral" sulphur is higher than the sum of the known substances belonging to this group (methionine, cystine and its derivatives, taurine, thiamine).

TABLE I

INORGANIC CONSTITUENTS OF HUMAN PLASMA OR SERUM

	Average or representative value mg. per 100 ml.	Range mg. per 100 ml.	References
Bicarbonate (as $NaHCO_3$)	226	205 to 280	2, 3
Calcium (serum)	10	8.2 to 11.6	2, 4
Chloride	365	355 to 381	3, 4
Copper	0.12	0.086 to 0.161	5, 6, 7, 8, 9
Fluorine (whole blood)	0.28		10, 11
Iodine (protein bound)		0.006 to 0.008	12, 13
Iron	0.105	0.028 to 0.210	5, 14, 15
Lead (whole blood)	0.03	0.009 to 0.05	16
Magnesium (serum)	2.0	1.7 to 2.3	17, 18, 19
Manganese (whole blood)		0.005 to 0.02	20
Phosphate (as P) (inorganic)	3.2	2.6 to 5.4	21, 22, 2
Phosphate (as P) (lipoid)*	8.0	6.1 to 9.9	21
Phosphate (as P) (ester)†	0.6	1.0 to 1.0	21
Phosphate (as P) (total)	12.1	10.0 to 14.1	21
Potassium	17.2	12.1 to 25.4	23, 24, 25, 26
Silica, soluble, whole blood (as SiO_2)	1.5		27
Silica, total, whole blood (as SiO_2)	9.0		27, 28
Sodium	316	300 to 330	23, 24, 25, 26
Sulphate (as S) (inorganic)	1.57	1.00 to 1.85	29, 30
Sulphate (as S) (ester)‡	0.39	0.25 to 0.65	29, 30
Sulphur (as S) (nonprotein, total)	3.38	2.95 to 3.75	29
Sulphur (as S) (neutral)§	1.42	0.90 to 1.95	29
Water	93600	92400 to 94400	33
Zinc	0.21	0.12 to 0.48	31, 32

* Organic phosphates soluble in organic solvents (ethanol-ether).

† Acid-soluble phosphates, excluding inorganic orthophosphate, liberating orthophosphate on acid hydrolysis.

‡ Organic substances liberating sulphate on acid hydrolysis (sulphuric acid esters of phenols and carbohydrate).

§ Substances giving sulphate on oxidation (S-containing amino acids, taurine, thiosulphate, thiocyanate, thiamine).

NITROGENOUS SUBSTANCES

Amino acids.—Microbiological assays and other sensitive methods have made it possible to measure quantitatively most of the individual amino

acids which constitute the amino nitrogen fraction of the plasma. All amino acids which commonly occur in proteins have been found to occur in the free form in plasma (or blood). The accuracy of the determination varies from compound to compound and is not always very great, but in most instances more than one method has been used and the results obtained by different methods and in different laboratories agree reasonably well. Representative data for normal human plasma are shown in TABLE II.

TABLE II

Amino Acids in Normal Human Blood Plasma

Amino Acid	Average or representative value mg. per 100 ml.	Range or standard deviation mg. per 100 ml.	References
Alanine	3.97	S.D. 0.70	36, 37
Arginine	2.34	S.D. 0.62	38, 39, 40
Citrulline	0.50	0.38 to 0.59	42
Glutamic acid	3.41	S.D. 1.39	43
Glutamine	5.78	S.D. 1.55	43, 44, 45
Glycine	1.77	S.D. 0.26	36
Histidine	1.42	S.D. 0.18	38, 40, 41
Isoleucine	1.60	S.D. 0.31	38, 40
Leucine	1.91	S.D. 0.34	38, 40
Lysine	2.95	S.D. 0.42	38, 40
Methionine	0.85	0.46 to 1.48	46, 40
Phenylalanine	1.38	S.D. 0.32	38
Threonine	2.02	S.D. 6.45	38, 40
Tryptophane	1.08	S.D. 0.21	38, 31
Tyrosine	1.48	S.D. 0.37	38
Valine	2.83	S.D. 0.34	38, 40, 41
Total α-amino N (as N, ninhydrin method)	4.1	3.4 to 5.5	47, 48
Total α-amino N (as N, nitrous acid method)	4.4	3.7 to 5.9	47

Glutamic acid and glutamine constitute about one-third of the total amino nitrogen and occur in much higher concentrations than any of the other amino acids; this is paralleled by their high concentration in many proteins, e.g., casein. In 54 samples of human blood the sum of glutamine and glutamic acid showed less variation than did the concentrations of either of the two components [Krebs, Eggleston & Hems (43)]. On the average 42.6 per cent of the sum was glutamic acid and 57.4 per cent glutamine. Reasons for the variation of the ratio of glutamic acid to glutamine were not apparent. Glutamine values obtained by other authors [Harris (49), Hamilton (50),

Archibald (51), Prescott & Waelsch (52)] are of the same order, but for glutamic acid the values of Prescott & Waelsch are somewhat lower than those obtained by Krebs *et al.*

Glycine and alanine contribute about another 24 per cent of the amino nitrogen of the plasma [Alexander (53)]. Of the other amino acids lysine is present in a relatively high concentration.

No data are as yet available on the concentrations of aspartic acid, serine, cystine, proline, and hydroxyproline in human blood plasma, but Wiss (54) has reported on the occurrence of these five amino acids in the whole blood of rats. Dent & Schilling (56), using a semi-quantitative chromatographic method, found aspartic acid, proline and serine in dog plasma. Hier (55) found cystine in dog plasma.

New determinations of the total α-amino nitrogen of human plasma by the ninhydrin method (47, 48) give somewhat lower values than previous determinations with the nitrous acid method. This is expected because of the greater specificity of the ninhydrin method. With the nitrous acid method glutamine yields 1.9 atoms of amino nitrogen per molecule and lysine 1.8, whilst both yield 1 atom per molecule with the ninhydrin method. On the basis of this difference the ninhydrin method should give values for the amino nitrogen which are about 0.7 mg. per 100 ml. lower. The actual difference is smaller, which may be due to the fact that proline and hydroxyproline react with ninhydrin but not with nitrous acid.

The average values of the 16 amino acids listed in TABLE II account for 3.93 mg. per 100 ml. plasma α-amino nitrogen as determined by the nitrous acid method, and for 3.26 mg. per 100 ml. as determined by the ninhydrin method. Bearing in mind that five amino acids, expected to be present in plasma, have not been included in the calculations because quantitative date are not available, it may be said that the value of the total α-amino nitrogen, calculated for the amino acids found, tallies reasonably well with the values obtained for the total α-amino nitrogen determinations (see TABLE II), a result which strengthens confidence in the data for the individual amino acids.

Considerable variations in the protein content of the food (40) have little effect on the amino acid content of human plasma. On rats, Wiss (54), analysing whole blood, observed some increases in the concentrations of the essential amino acids when the animals were kept on a protein-rich diet, whilst in contrast the concentration of the nonessential amino acids was lower on a protein-rich diet than on a protein-low diet. Temporary increases in the concentrations of individual amino acids occur during the period of absorption after feeding (36, 40, 55, 56). It is remarkable that the rise in the plasma concentration of the amino acid ingested in some cases [when leucine, isoleucine, methionine (55), or glutamic acid (57) is given] is accompanied by a fall in the level of other amino acids.

Such data as are available suggest that the concentrations of the various amino acids in the plasma are very similar in different mammalian species (38).

Conjugated amino acids (peptides).—Deproteinised filtrates of plasma have long been known [Hiller & Van Slyke (58)] to contain conjugated amino acids which are set free on hydrolysis. The findings of 11 previous investigators, summarized by Christensen & Lynch (59), showed considerable discrepancies, values ranging from 0 to 7 mg. per 100 ml. nitrogen. These discrepancies may have been due to differences in the technique of deproteinisation. Christensen & Lynch (59) reinvestigated the concentration of the "combined non-protein α-amino-N" and found values between 0.1 and 2.2 mg. per 100 ml. (average 0.9 mg. per 100 ml.; S. D. = 0.44) for fasting human adults. There was little difference between tungstic and and trichloroacetic (2.5 per cent) filtrates. Clotting of plasma caused increases of 1 to 3 mg. per cent in trichloroacetic acid filtrates, but none in tungstic acid filtrates. Christensen & Lynch report data on the glycine and alanine content of the peptides and Sheffner *et al.* (40) on the proportion of leucine, isoleucine, valine, threonine, arginine, histidine, lysine and methionine.

Serum deproteinised with 0.6 *M* perchloric acid or 0.2 *M* sulphosalicylic acid, or by heat coagulation at pH at 4.7, contains a nondialysable "proteose" which, on hydrolysis, yields amino acids and glucosamine. The average concentration of this "serum mucoid" or "mucoprotein" in normal human adults according to Winzler *et al.* (60), is 86.7 $\pm$ 9.5 mg. per 100 ml. and is raised in cases of cancer and pyogenic infection [see also (61, 62, 63)]. The substance contained on the average 3.9 per cent tyrosine, 6.5 per cent nitrogen, and 15 per cent carbohydrate (60). Cystine and tryptophane were also present (62). "Serum mucoid" is precipitated by tungstic acid and high concentrations (0.5 *M*) of trichloroacetic acid (60).

Creatine and related substances.—The older quantitative data on the creatine and creatinine content of the body fluid were mainly based on the somewhat unspecific Jaffé reaction between creatinine and alkaline picrate. The use of a specific bacterial enzyme capable of destroying creatine and creatinine (64, 65) made it possible to determine these two substances with greater accuracy, applying the Jaffé reaction before and after the enzymatic destruction of creatine and creatinine. Allinson (66) found that about 70 per cent of the "apparent" creatine and about 85 per cent of the "apparent" creatinine in plasma and serum were "true" creatine and creatinine. Tierney & Peters (67), using an improved photoelectric method based on the Jaffé reaction (68), found values of a similar order. Hoberman (39) found that glycocyamine, the physiological precursor of creatine, is a regular constituent of blood plasma. Data are shown in TABLE III.

Survey of the composition of the nonprotein nitrogen.—Newer data on substances belonging to this fraction for which quantitative information is available and which have not yet been discussed, are included in TABLE III. They do not warrant detailed discussion except for the contribution which they make to the nonprotein nitrogen. The average values of the various substances previously given in terms of mg. substance per 100 ml. have been converted into mg. nitrogen per 100 ml. and are shown in TABLE IV. The amino acid nitrogen, as opposed to α-amino nitrogen, calculated from the

TABLE III

DATA ON THE CONCENTRATION OF COMPONENTS OF THE NONPROTEIN NITROGEN FRACTION OF PLASMA OR SERUM (OTHER THAN AMINO ACIDS AND PEPTIDES)

(Human adults unless otherwise stated)

Substance	Average or representative value mg. per 100 ml.	Range or standard deviation mg. per 100 ml.	References
Creatine and related substances			
Creatine (serum)* (as creatinine)	0.42	0.28 to 0.62	66
Creatine (serum) male†		0.17 to 0.50	67, 68
Creatine (serum) female†		0.35 to 0.93	67, 68
Creatine (serum)*	1.07	0.76 to 1.28	66
Creatinine (serum) male†		1.05 to 1.65	67, 69, 70
Creatinine (serum) female†		0.90 to 1.50	67, 69, 70
Guanidino acetic acid	0.26	0.24 to 0.28	39, 69
Purine derivatives			
Uric acid (serum)	4.0	2.9 to 6.9	71, 72
Allantoin		0.3 to 0.6	42
Allantoin (dog)		1.1 to 3.0	42
Adenosine plus adenylic acid (as adenosine)‡	1.09	S.D. 0.385	73
Other substances			
Urea, male	27.1	S.D. 4.5	48 (compare 74, 75)
Urea, female	26.4	S.D. 8.1	48 (compare 74, 75)
Ammonia (as N)	below 0.05		76, 77
Choline§		0.3 to 1.5	77a

* Method employing creatine destroying enzyme (64, 65).
† Photoelectric method.
‡ Measured by guinea pig atrium assay.
§ Low values found in July, high values in February and March.

nitrogen content of the amino acids listed in TABLE II, gives a value of 5.0 mg. nitrogen to which an arbitrary amount of 1.0 mg. has been added for the five amino acids not listed but known to be present. An arbitrary factor of 1.33 has been used to convert the α-amino nitrogen of conjugated amino acids into total conjugated amino acid nitrogen.

About one-half of the nonprotein nitrogen comes from urea and one-quarter from free and conjugated amino acids (48). The sum of the nitrogen of all these substances is rather less than the total nonprotein nitrogen, the deficit being 3.3 mg. per cent or 12 per cent of the nonprotein nitrogen.

However, there is an uncertainty in this figure, as the calculations are based on average values obtained by different investigators. It is possible that the unknown fraction is in reality smaller.

TABLE IV

AVERAGE COMPOSITION OF THE NONPROTEIN NITROGEN FRACTION OF HUMAN PLASMA AND SERUM

[All substances expressed as N; the data for nonprotein nitrogen and urea are quoted from (48)]

Substance	mg. N per 100 ml.	
Total nonprotein nitrogen	25.7	
Urea	12.7	=22.4
Free amino acids	6.0	
Conjugated amino acids	1.2	
Creatine, creatinine, guanidino-acetic acid	0.7	
Uric acid, allantoin	1.5	
"Adenosine"	0.3	

Plasma proteins.—The progress in the fractionation of plasma proteins has been reviewed in a recent volume of this series (78) and need not be discussed in full. Only quantitative aspects of the concentration of the various plasma protein components are considered here. Data are given in TABLES V and VI, the former listing the concentrations of the main six electrophoretic fractions, the latter the main components obtained by the Harvard School (82, 83) with the ethanol fractionation method. Of the 25 fractions or more, which Cohn and his team (82, 83) have separated, those for which

TABLE V

PLASMA PROTEINS

Representative values for the concentration of electrophoretic fractions of human plasma protein [Dole (79); see also (80, 81)]

Protein	gm. per 100 ml. plasma	
	Mean	S.D.
Albumin	4.04	0.27
α_1Globulin	0.31	0.051
α_2-Globulin	0.48	0.053
β-Globulin	0.81	0.126
γ-Globulin	0.74	0.151
Fibrinogen	0.34	0.059
Total	6.72	

TABLE VI

DATA ON THE CONCENTRATION OF COMPONENTS OF HUMAN PLASMA PROTEINS

[Modified after Cohn (82, 83)]

Substance	Characteristic Properties	Assumed Functions	Estimated Amount: gm. in 100 gm. plasma protein	Estimated Amount: gm. in 100 ml.* plasma	Electrophoretic Fraction in which Protein occurs
Fibrinogen	Conversion into fibrin	Blood clotting	4	0.27	
Antihaemophilic globulin	Clots haemophilic blood	Blood clotting	very little		
Non-clottable protein	Insoluble at low temperature		0.15	0.01	
Immune γ-globulin	Antibodies for diphtheria, measles, mumps, influenza, pertussis, streptococci antitoxin, typhoid-H-agglutinins	Immunological	11	0.74	γ-globulin
Immune euglobulins	Typhoid "O" agglutinins	Immunological	very little		β- and γ-globulins
Isoagglutinins	Anti-A, anti-B and anti-Rh antibodies	Immunological	(0.03)	(0.002)	β- and γ-globulins
Complement components	Lysis	Immunological	0.4	0.027	α- and β-globulins
Enzymes	Thrombin, proteolytic, amylolytic, lipolytic enzymes, phosphatase (alkaline), cholinesterase	Metabolic	0.02	0.001	α- and β-globulins
β-Pseudoglobulin, (crystallised)	Combining with Fe and Cu	Solubilisation and transport of specific substances	2.5	0.17	α- and β-globulins
Protein combining with iodine		Solubilisation and transport of specific substances	very little		α- and β-globulins
Glycoproteins	Containing carbohydrate (mucoproteins)		1.2	0.08	α- and β-globulins
Lipoproteins	Combined with steroids and carotenoids	Solubilisation and transport of specific substances	8	0.04	α- and β-globulins
Bilirubin-containing proteins			0.05	0.003	α-globulin
Albumin (crystallised)	Combines with fatty acids, bile salts, bile pigments, iodine, dyes and drugs	Solubilisation and transport of specific substances and osmotic regulation of blood volume	50	3.35	albumin

* Calculated for a total plasma protein of 6.7 gm. per 100 ml.

quantitative information on the concentration is available are included in TABLE VI. Two of the fractions named are crystalline, the others must be regarded as highly complex mixtures. The substances listed as being present in these complex fractions probably present no more than a small proportion of the fractions. Nevertheless it is a noteworthy advance that many of the substances which, on account of their activity as antibodies, hormones, and

TABLE VII

Low Molecular Non-Nitrogenous Intermediary Metabolites in Human Plasma

Substance	Average or representative value mg. per 100 ml.	Range or standard deviation mg. per 100 ml.	References
Intermediates of the tricarboxylic acid cycle			
Citric acid	2.5	1.9 to 2.8	84, 85
α-Ketoglutaric acid	0.8		86
Succinic acid	0.5		87
Malic acid (whole blood)	0.46	0.24 to 0.75	89
Fumaric acid (whole blood)	<0.3		90
Ketone bodies			
Acetoacetic acid (non-fasting)		0.8 to 2.8	91
Total, as β-hydroxybutyric acid (non-fasting)	0.5	0.3 to 0.9	92
Miscellaneous substances			
Lactic acid (resting)		8 to 17	93
Pyruvic acid	1.0	0.77 to 1.23	94, 95, 91
Total α-ketonic acids (as pyruvic acid)		0.6 to 2.1	96
Volatile fatty acids (as acetic acid), whole blood	1.8		97, 98, 98a

enzymes are known to occur in plasma, have been located in well-defined fractions of the plasma protein.

Low Molecular Intermediary Metabolites (Non-Nitrogenous)

Intermediates of the tricarboxylic acid cycle.—Of the nine di- and tricarboxylic acids, which occur as intermediates in the tricarboxylic acid cycle, five have been detected and quantitatively determined in the plasma or blood. They are citric, α-ketoglutaric, succinic, malic, and fumaric acids (TABLE VII). The concentration of each of the first four substances is of

the same order, varying between 0.5 and 2.5 mg. per 100 ml.; the concentration of fumaric acid is somewhat lower. The main reason for the failure to demonstrate the other intermediates is probably the lack of sensitive methods of analysis. The first to be found was citric acid [Thunberg (88)]. Its concentration under a variety of conditions has been frequently studied following upon the pioneer work of the Thunberg School. Increases occur in diabetes mellitus and various other diseases.

Ketone bodies.—The values (see TABLE VII) for acetoacetic acid given by Rosenthal (91) are somewhat higher than those for total ketone bodies given by Weichselbaum & Somogyi (92). This discrepancy may be connected with differences in the nutritional state of the subjects examined. Fasting has a greater effect on the concentrations of the ketone bodies than on those of any other plasma constituent. In rat and mouse plasma Rosenthal found the ketone bodies increased more than ten-fold after 24 hr. fasting. After 48 hr. fasting mouse plasma was found to contain 290 mg. acetoacetic acid per 100 ml.

Miscellaneous substances.—There has been no major new development in the knowledge of the level of lactic acid and its changes under varying conditions. The occurrence of a characteristic level of pyruvic acid, increased by exercise and in vitamin-B_1 deficiency, is now well established. Of the total α-ketonic acids, as determined by the yeast carboxylase method [Westerkamp (96)], by far the main components are pyruvic and α-ketoglutaric acid. Volatile fatty acids, probably mainly acetic acid, have been determined in human blood by McClendon (97) and Phillipson (98).

Carbohydrates and related substances.—Of the substances listed under this heading in TABLE VIII only glucose, and possibly traces of pentose, occur in the free state. "Polysaccharides" and "glucosamine" occur in combination with protein and hexuronates are present as esters.

The values for "polysaccharides" and "glucosamine," although well reproducible, do not represent any one precisely defined substance, but rather groups of substances giving some more or less specific quantitative tests. The "polysaccharide" determined by the carbazol method is presumed to be composed approximately of equimolecular quantities of galactose, mannose, and glucosamine [Hewitt (113)]. The "polysaccharides" determined by the tryptophane method do not include the glucosamine fraction (112). It is clear that the values given for the two polysaccharides and glucosamine refer in part to the same substance. They also overlap with the "serum mucoid" mentioned earlier in this review. With the techniques employed, values for normal adults are consistent and keep within a limited range. Higher values are regularly found in infectious and malignant diseases (81, 114). The pentose values, as determined by the Mejbaum reaction (115), may be looked upon as indicative of nucleosides and nucleotides in plasma.

Although numerous papers continue to be published on blood sugar, only a few data are available on the glucose concentration in human plasma or serum. However, the differences between plasma glucose and whole blood glucose may be taken as small in normal humans and monkeys (116). They

are considerable in most other species where the concentration within the cells is much lower than in the plasma (116).

Fat and related substances.—The main groups of lipids which occur in the tissues are also found in the plasma, with the exception of the cerebrosides

TABLE VIII

CARBOHYDRATES, FATS, AND THEIR DERIVATIVES IN HUMAN PLASMA

Substance	Average or representative value mg. per 100 ml.	Range or standard deviation mg. per 100 ml.	References
Carbohydrates and related substances			
Glucose, fasting, venous whole blood	83	S.D. 4	99, 100
Glucose, fasting, capillary whole blood	93	S.D. 3	99, 100
Polysaccharides, serum (as hexose)*	102	73 to 101	101, 81
Polysaccharides ("non-glucosamine")†	110	93 to 125	102
Glucosamine (total)	77	63 to 88	63, 102
Hexuronates (as glucuronic acid)‡		0.4 to 1.4	103
Pentose, total§	2.55	0.37	73, 104, 105
Pentose, phosphorylated‖	2.19	0.30	73, 104, 105
Fat and related substances			
Fatty acids, total (as stearic acid)		200 to 450	106
Neutral fat		0 to 150	106
Cholesterol, free		40 to 70	106, 107
Cholesterol, total		150 to 260	106, 107
Phospholipids, total		150 to 250	106, 108
Lecithin		100 to 200	106, 108, 109, 110
Cephalin		0 to 30	106, 108, 109, 110
Sphingomyelin		10 to 30	106, 108, 109, 100
Bile acids (as cholic acid)		0.2 to 3.0	111

* Substances other than glucose giving a colour reaction with carbazol in the undeproteinised serum (101)

† Substances other than glucose giving a colour reaction with tryptophane (112)

‡ Substances giving colour reaction with napthoresorcinol

§ Includes free, nucleoside and nucleotide pentose

‖ Mainly nucleotide-pentose

which are essentially intracellular lipids. The plasma level of all lipids is very variable, as will be seen from concentration ranges quoted as normal in TABLE VIII. Characteristic deviations from the wide normal range have

been recorded under pathological conditions for all groups of substances except the sphingomyelin fraction [see Thannhauser (106)].

Substances of Special Physiological Activity

Vitamins.—Detailed information on the concentration of vitamins in plasma is still limited. Data referring to well-nourished human adults are shown in TABLE IX. In general the plasma concentration of vitamins is rather lower than the concentration in the tissues, and the level of vitamins

TABLE IX

Vitamins in Human Plasma

Vitamin	Average or representative value mg. per 100 ml.	Range mg. per 100 ml.	References
Fat-soluble			
Vitamin A	0.025	0.019 to 0.036	117, 171a, 119
Carotene (total carotenoids)	0.09	0.06 to 0.18	117, 117a, 119
Vitamin E (tocopherols)	1.20	0.90 to 1.59	120, 121, 122, 123
Water-soluble			
Ascorbic acid		0.1 to 0.70	124
Thiamine	0.0005		125
Nicotinic acid plus nicotinamide	0.03	0.02 to 0.05	126, 127, 128
Inositol		0.42 to 0.76	129
Folic acid	0.00175	0.00162 to 0.00195	130, 131
Biotin	0.00127	0.00095 to 0.00166	130
Pantothenic acid	0.012	0.006 to 0.022	130
Riboflavin plus flavin-mononucleotides, expressed as riboflavin	0.0008	0.0003 to 0.013	132
Total riboflavin	0.0032	0.0026 to 0.0037	132

in plasma is much more variable than the level of many other plasma constituents, the dietary intake having a great influence on the plasma vitamin level. For example, the concentration of carotene fell in one series of observations from the level of about 0.09 mg. per 100 ml. to almost nil within four weeks, when carotene was withheld from the diet [Hume & Krebs (117)]. With (118) states that in European populations the total carotenoid content seldom exceeds 0.1 mg. per 100 ml., whilst Kirk & Chieffi (119) report for North American populations average total carotenoid values of 0.2 to 0.3 mg. per 100 ml. The ascorbic acid content of plasma likewise depends on the ascorbic acid supply and if the intake is below 20 mg. daily (10 mg. is enough

to prevent scurvy) (124) the plasma value of the vitamin falls to below 0.10 mg. per 100 ml. (124). With present methods of analysis such concentrations are difficult to distinguish from that found in scorbutic patients (124). The correlation of ascorbic acid intake and plasma level is shown in TABLE X. Similarly riboflavin and its derivatives have also been shown to disappear

TABLE X

PLASMA ASCORBIC ACID CONTENT OF FASTING HUMAN ADULTS IN RELATION TO ASCORBIC ACID INTAKE

Approximate intake mg. daily	Average ascorbic acid in plasma mg. per 100 ml.
0	<0.03
5	<0.05
10	<0.10
20	<0.10
50	0.31
70	0.55
600	1.02

rapidly from the plasma when the intake is curtailed (132). In contrast the level of vitamin A is fairly constant and independent of daily fluctuations of the vitamin A intake. The exceptional position of vitamin A is probably connected with the fact that in normally fed individuals the liver stores a large amount of the vitamin, sufficient to cover the requirements for a period of from six months to two years (117). Presumably this store maintains the constant plasma level.

Hormones.—Most hormones are carried in the blood plasma from the site of their production to that of their action, but only for a few has it so far been possible to measure their concentration in the plasma. Some data, which should be regarded as provisional, are shown in TABLE XI. The reli-

TABLE XI

HORMONES IN HUMAN PLASMA

Hormones	Range mg. per 100 ml.	References
Epinephrine (whole blood)	0.0041 to 0.0096	133, 134, 138
Corticosteroid	0.11 to 0.42	139, 140
Oestrogen (as oestriol equivs.)	0.0002 to 0.0022	141
Progesterone	0.5 to 0.8	142

ability of the epinephrine value given by Jørgensen (133) has been questioned by Bloch 134). Lehmann & Michaelis (135, 136) report much higher epinephrine values (0.2 to 1.0 mg. per 100 ml.) but admit that "epinephrine," as

determined by their fluorometer procedure, is physiologically largely inactive [see also (137)].

Enzymes.—The presence of many enzymes, in fact of most hydrolytic enzymes which occur intracellularly in animal tissues, has been demonstrated in plasma and serum. Enzymes concerned with oxido-reduction are, as a rule, absent from plasma, but Warburg & Christian (143) have demonstrated in the plasma of rats five of the enzymes taking part in the conversion of glucose into lactic acid, viz., zymohexase, isomerase, the protein component of lactic dehydrogenase, and two of the phosphorylases.

Most of the information on plasma enzymes is of a qualitative or semiquantitative nature, and such quantitative data as are available refer to relative activities rather than to absolute enzyme quantities. The one exception is zymohexase which Warburg & Christian (143) have determined in rat serum. They found a value of 0.028 mg. per 100 ml. plasma in normal animals and a 10 to 20-fold increase in animals carrying large malignant tumours. On the assumption that the quantities of other enzymes in the plasma are of a comparable order and that only, say, 10 per cent of the serum protein has enzymatic properties, serum would be capable of harbouring hundreds of enzymes.

Many measurements of enzyme activities in plasma, expressed in arbitrary units, are available for acid and alkaline phosphatases, cholinesterases, amylases, lipases, and various proteolytic enzymes. These cannot be discussed in full in this review.

Pigments

Bilirubin is the only plasma pigment which is known to occur at a regular level of concentration, the normal average for human plasma being 1.0 mg. per 100 ml. (144, 145, 146). The amounts of carotenoids in the plasma, as already mentioned, depend on the presence of these substances in the diet. In addition to bilirubin and carotenoids, plasma contains other yellow pigments in quantities comparable to that of bilirubin (as judged by their extinction coefficient). Their chemical nature is uncertain [see 118, 144, 147)].

Special Physiological Conditions

The data so far discussed generally refer to normal adults. Deviations due to age or special physiological circumstances cannot be dealt with fully, but a few of the more recent advances are briefly mentioned.

Age.—A striking difference in the composition of plasma related to age is the replacement of the major part of the glucose by fructose in foetal plasma of ruminants, sheep, ox, and goat (148 to 151). Whole blood and plasma of sheep foetuses between the 72nd and 144th day of pregnancy were found to contain between 54 and 134 mg. per cent fructose (149), the value being generally closer to the lower end of the range towards term. Soon after birth the fructose is exchanged for glucose. In one observation reported by Cole & Hitchcock (149) 27 mg. per 100 ml. fructose were found in the blood 1 hr. after birth, and 3 mg. per 100 ml. after 24 hr. Many data on lesser differences

between foetal and maternal blood have been collected by Needham [(152); see also (153)]. In postnatal life a major deviation from the normal adult occurs in the case of inorganic phosphate, which in children may amount to over 7 mg. per 100 ml. (as phosphorus). Simonsen *et al.* (22) found an average value of 5.7 mg. phosphorus per 100 ml. in children between 3 and 13 years. In their group of subjects the average serum phosphate of children was 1.8 mg. per 100 ml. higher than that of adults. The differences disappear at the age of 19 or 20 [Stearns & Warweg (21)], i.e., at the time when the calcification of the bones approaches completion.

McCance & Widdowson (154) observed a temporary increase in the urea level on the first days of life, the average urea concentration being 18.7 mg. per 100 ml. at birth, 29.1 mg. after 3 to $3\frac{1}{2}$ days and 19.7 mg. after 6 to 62 days. This seems to result from a temporary limitation of kidney function due to hydropenia (154). The blood sugar level is low is newborn infants and rises within the first few days of life. Norval *et al.* (155) found a mean value of 56.7 mg. per 100 ml. whole blood (S. D. 16.4) on the first day of life. The average rise per day was 2.8 mg. per 100 ml. during the first six days. The plasma chemistry of the first days of life has by no means been fully studied and future work is likely to reveal further differences.

A small rise in the average non-protein nitrogen level in old age has been reported by Olbrich (156). With reference to most substances it may be said that generally there is a remarkable constancy in the composition of plasma from early childhood to old age.

Exercise.—The main change in the plasma caused by exercise is the rise in the lactic acid concentration and to a smaller degree in the level of pyruvic acid. Goldsmith (157) found that an average fasting lactic acid level of 9.3 mg. per cent rose to an average of 35 mg. per cent on strenuous exercise. Under the same conditions the pyruvic acid level rose from 1.00 to 1.96 mg. per cent. Mild exercise had no appreciable effects.

Fasting.—On short term fasting the most pronounced change is the rise of the concentration of ketone bodies already briefly referred to. This rise varies considerably from species to species. Kartin *et al.* (158) found in human subjects an average of 0.4 mg. per 100 ml. initial ketone bodies (as acetone), which after two days fasting rose to 11.1 mg; after five days values up to 37 mg. were recorded. In monkeys the rise was greater than in man, whilst dogs did not develop appreciable ketosis (158). Among rodents ketosis on starvation is more severe in rats and mice than in guinea pigs (91).

Sunderman (159) studied changes in the plasma of a man who fasted 45 days. At the end of this period the chloride and glucose levels were lowered by about 25 per cent, whilst an increase was found for bicarbonate (60 per cent), magnesium (40 per cent) calcium (25 per cent) urea (100 per cent), creatinine (40 per cent), and uric acid (120 per cent).

Posture.—New data on the effects of posture on the plasma protein concentration have been published by Spealman *et al.* (160). On the average the plasma protein rose from 6.7 to 7.5 mg. per 100 ml. in 30 min. after changing from the lying to the standing position [see also (160a)]. This phenomenon

has been investigated previously [e.g., (161)] but seems to be frequently ignored in clinical and nutritional studies of plasma protein levels. Coniglio (162) noticed a drop of 30 to 60 per cent in the inorganic phosphate of dog plasma when the nonanaesthetised animals were restrained in the supine position.

Pregnancy.—On a percentage basis the increase in the concentration of plasma copper is the most pronounced change so far known to occur during pregnancy (157, 158, 163). The increase begins in the third month (163) and reaches on the average a level about 100 per cent above the normal range. After delivery it decreases rapidly and reaches the normal level again within a few weeks (163). The plasma level of inorganic iron does not change in normal pregnancy (164).

Relatively small changes have been reported for several plasma constituents. Bonsnes (165) found a decrease in the α-amino nitrogen as determined by the ninhydrin method, the average being 3.2 mg. per cent during pregnancy and labour against a normal value of 4.3 mg. per cent. Heinemann *et al.* (166) found an increase in the serum precipitable iodine, the range being 6.2 to 11.2 μg. per cent in normal pregnancies, against 4.0 to 8.0 in normal nonpregnant persons. The elevation was noted as early as three weeks after conception; it did not increase as pregnancy advanced and decreased rapidly to the normal range after delivery. Hoch & Marrack (164) report a small decrease in the total serum protein with approximately constant globulin level.

Some Species Differences

It is not possible to compare here findings on different species in detail. It may be said that the concentration of many plasma constituents shows a striking uniformity in different mammalian species. This is particularly true for most of the inorganic ions [see Pasquier (167)]; it also applies, though less rigidly, to most components of the nonprotein nitrogen fraction, to most trace elements and, in the majority of species, to glucose.

A few species differences are shown in TABLE XII. Noteworthy are the low sulphate content of human plasma (30) (compare data in TABLES I and XII), the differences in the concentrations of uric acid and allantoin, arising from the variations in the occurrence of uricase in different species, and the low glucose content of ruminant blood (168, 169). As the red cells of ruminants contain much less glucose than the plasma (100, 170), the glucose level of plasma is somewhat higher than that of whole blood. But even if an allowance is made for this, the glucose plasma level in ruminants is considerably lower than that of man and other mammals (170).

The plasma of birds, reptiles and amphibia markedly resembles mammalian plasma, though characteristic differences in metabolism, such as a uricotelic or ureotelic nitrogen metabolism, are reflected in the concentrations of the nitrogenous metabolites in the plasma. The concentration of urea in fowl plasma is only about one-twentieth of that in mammalian plasma (172). The fasting glucose level in birds is generally higher than in mammals

(TABLE XII). The plasma protein value of birds is generally lower than that of mammals (175). Werner (175) found in female ducks a much higher plasma protein level (average 6.5 gm. per 100 ml.) than in males (average 4.5 gm. per 100 ml.), and suggests that this may be connected with egg production.

Considerable species differences exist with regard to the activities of the enzymes and enzyme inhibitors of plasma. For example, the cholinesterase

TABLE XII

SPECIES DIFFERENCES IN THE COMPOSITION OF PLASMA

(Compare with data for man shown in TABLES I, III AND VIII)

Substance	Species	Average or representative value mg. per 100 ml.	Range or standard deviation mg. per 100 ml.	References
Glucose (whole blood)	Cattle	49.2	S.D. 6.3	168
Glucose (whole blood)	Sheep	44	21 to 61	169, 170
Glucose (whole blood)	Birds		81 to 347	171
Urea	Fowl	1.5		172
Uric acid	Rat	1.5	0.5 to 3.4	173, 174
Allantoin	Dog		1.5 to 3.0	42
Allantoin	Rat	2.1	0.6 to 3.2	173
Sulphate (as S) (inorganic)	Dog	3.2		30
Sulphate (as S) (inorganic)	Horse	3.4		30

activity of horse plasma is about 70 times greater than that of human plasma (176). Asparaginase activity of guinea pig plasma is at least 200 times greater than that of human plasma (177, 178). There is an inhibitor of carbonic anhydrase in the plasma of the pig, the sheep, the horse, the ox, the cat, and the rat, but not in the plasma of the monkey, the duck, the pigeon and of man (179). Many more examples of such differences could be quoted. Their physiological significance is still obscure.

In surveying the recent work as a whole, one is struck by the wealth of new data and the spectacular advances in techniques.

Quantitative information is available for over 90 substances in human plasma. Yet there can be no doubt that many other substances will have to be added to the list, especially as further advances are made in the resolution of the protein fraction, which is known to contain a large number of different enzymes, hormones, antibodies, and anti-enzymes.

In many cases only a few micrograms of material are now required for analysis as a result of new microbiological and optical procedures and the development of micromanipulation (180 to 183). Hence a fraction of a drop

of plasma may now suffice for an accurate analysis, and this opens up new approaches to many problems in biology and medicine.

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PYRROLE PIGMENTS

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Introduction

In the two years which have elapsed since the publication of Lederer's review (1) several workers prominent in the field of pyrrole pigments have produced authoritative reviews of their special subjects (2 to 11). Fitting tribute to the memory of Sir Joseph Barcroft has been paid at a Memorial Meeting at Cambridge, 1948, the proceedings of which, edited by Roughton & Kendrew, are now available (12). The death of L. Michaelis has removed another prominent worker.

The demonstration, by means of labelled glycine, that the synthesis of the porphyrin nucleus and its incorporation into haemoglobin proceeds in the avian erythrocyte *in vitro*, has opened a new field of research. The elucidation of the biosynthesis of porphyrins now appears a distinct possibility. The high N^{15}-content of stercobilin soon after feeding N^{15}-glycine is an unexpected discovery, which raises important problems.

The two years under review have also brought important advances in our knowledge of the mode of action of catalase and peroxidases on hydrogen peroxide and of their kinetics, progress in the isolation of haemin *a* and a renewed attack on its structure, the discovery of a new haematin factor in cellular respiration, classification of the structure of intermediates between haematin compounds and bile pigments, the isolation of magnesium porphyrins from *Chlorella* mutants, and the use of paper chromatography for the study of porphyrins.

Nomenclature.—We propose to use a modified nomenclature which we have discussed elsewhere (4, pp. 164, 208), together with appropriate acknowledgements to its various originators. Use is made of the familiar "o-i" changes denoting oxidation state in haem*o*chrome-haem*i*chrome and haem*o*globin-haem*i*globin, the "-chromogen-" ending being shortened to "-chrome." Our systematic bile pigment nomenclature (4, p. 105) is a simplified version of that developed by the Fischer school and similar to that adopted by Granick & Gilder (3).

Porphyrins

Sveinsson, Rimington & Barnes (13) have refined the procedures by which porphyrins may be isolated from porphyria urines, while Nicholas & Rimington (14) have published their procedure for the paper-chromatographic separation of the porphyrins. Lutidine-water is used as the mobile phase in an atmosphere containing ammonia vapour. The R_F is inversely proportional to the number of carboxyls present, uroporphyrin (eight carboxyls) migrating least and phylloerythrin (one carboxyl) furthest. The introduction of metal

had no effect, and monazaprotoporphyrin was not separable from protoporphyrin, nor uroporphyrin I from uroporphyrin III. In addition to uroporphyrin and coproporphyrin an unknown porphyrin with five to seven carboxyls was found in the urine of congenital porphyria. No such porphyrin was observed, however, in the urine of acute porphyria, or in the uroporphyrin III isolated from it. This appears to contradict earlier findings of Watson that uroporphyrin III is a mixture of uroporphyrin I with a heptacarboxylic porphyrin of type III. McSwiney, Nicholas & Prunty (15), however, found a porphyrin with probably five carboxyls (m. p. of ester 227°), and traces of another porphyrin, probably with seven carboxyls, in a case of acute porphyria. Nicholas & Comfort (16) found no evidence to support the claim of Fischer that the "conchoporphyrin" of *Pteria radiata* contained five carboxyl groups. Fresh material, as well as a sample of the original porphyrin prepared by Fischer & Jordan, gave only a mixture of uroporphyrins and coproporphyrins. The distribution of porphyrins in molluscan shells has been studied by Comfort (17). The porphyrin produced by *Corynebacterium diphtheriae* is coproporphyrin III (18); considerable amounts of this porphyrin have also been isolated from *Mycobact. Karlinski* (186). Klüver (19) has demonstrated the presence of porphyrins of urotype or coprotype in the root nodules of legumes. Studies on erythrocyte protoporphyrin have been reviewed by Cartwright & Wintrobe in last year's *Annual Review of Physiology* [(10), *cf.* also (20)].

Bile Pigments and Dipyrrolic Substances

The structure of the main bile pigment classes has now been established and a satisfactory correlation between structure and properties, particularly between the number of conjugated double bonds and colour, has been obtained [*cf.* (3, 4)].

It is now abundantly clear that urinary urobilin and faecal stercobilin contain a mixture of the same two pigments, mesobiline-(b) and tetrahydromesobilene-(b), with the latter normally prevailing, and the use of the terms "urobilin" and "stercobilin" for these two substances respectively is therefore misleading. Still other urobilinoid pigments may be present. A "third bile pigment" has been claimed to be present in infected bile by Meyer (21) and by Eisenreich (22); the description of its properties is too scanty to allow a comparison with Watson's "d-urobilin" (23), in fact it may not be a single substance. Whereas Watson assumed mesobilan to be an intermediate in the reduction of bilirubin to tetrahydromesobilan by intestinal bacteria, Baumgärtel (24) and Eisenreich (22) believe that mesobilan is of different origin and is formed by tissue enzymes.

Watson (25) has reviewed the bile pigments and porphyrins in jaundice and liver diseases. It has been reported that values of bilirubin in umbilical cord blood in excess of 4 mg. per 100 ml. are an early sign of erythroblastosis foetalis (26).

Bile pigment-proteins.—Blinks, Haxo & Yocum (27, p. 525) have shown

that light absorbed by the bile pigment-proteins, phycoerythrin and phycocyanin, is the only one used for photosynthesis in red algae, whereas light absorbed by chlorophyll is ineffective. Cohn (28) found that one special fraction (V—1), forming only 1/50 of the total of the serum albumin fraction, is a definite bilirubin-protein. It gives the "indirect" van den Bergh reaction. In view of the protracted controversy on the nature of "direct" and "indirect" bilirubin [*cf.* (1, 4)], future work will be required before this particular protein can be considered solely responsible for the "indirect" reaction. Two to three molecules of bilirubin combine with one molecule of serum albumin (28, 29) and may be dialysed off at low pH. According to Martin (29) and Gray & Kekwich (30), bilirubin is also able to combine with α_1 and α_2 globulins, migrating with these proteins during electrophoresis.

Bile pigments in invertebrates.—Comfort (17, 31) has commenced an extensive study of molluscan shell pigments. Whereas the turboglaucobilin isolated by Tixier (32) is a bilatriene closely related to (or identical with) coprobiliverdin, a variety of pigments of a different type are also present, as well as porphyrins. The nature of the haliot violin of *Haliotis cracherodii* is still uncertain. Whereas Comfort (17) has confirmed the similarity of its absorption spectrum with that of indigo, the analytical figures of Tixier & Lederer (33) rather suggest that the pigment is of tetrapyrrolic nature, although not a biliviolin.

Bilifuscins and pentdyopents.—The structural relationships between these dipyrrolic derivatives are not yet clearly established (4, 34 to 37). "Pentdyopents" are derived from colourless propentdyopents, which are formed by oxidation of haematin compounds or bile pigments with hydrogen peroxide; the propentdyopents are probably dipyrrylketones. "Pentdyopents" with vinyl side chains (adsorption band at 535 mμ) and with ethyl side chains (absorption band at 525 mμ) can be formed (38), a fact which strikingly reveals the inadequacy of this nomenclature. Siedel *et al.* (35) have now found that bilifuscins and mesobilifuscins are also derived from colourless "probilifuscins" or "bilileukans," which can be formed by the action of oxygen in the presence of reducing agents (sodium-amalgam, tissue) on bile pigments. They appear to be dipyrrylmethane derivatives. Polymerisation to the brown amorphous bilifuscins is catalysed by hydrogen ions. Faeces contain apparently both promesobilifuscin and bilifuscin. The importance of these observations for the balance of haemoglobin catabolism will be discussed in connection with the latter.

Haematin Compounds

Clark *et al.* (39, 40) have continued their potentiometric and spectrophotometric studies on haematin compounds with bases such as pyridine, and with cyanide. In the absence of nitrogenous bases haem and haematin exist mainly as dimers in solution, although the equilibrium between them has the characteristics of a one-electron change ($n=1$). The bonding of the two units which form the dimeric unit cannot too intimately concern the iron. The reaction of protohaematin with hydroxyl ion has a pK of 7.4 to

7.6. The dimers split on combining with cyanide ion or pyridine, with the exception of the combination of haematin hydroxide with pyridine, in which only one mole pyridine is added per mole of iron and where the structure remains dimeric. The structure of this compound is still not satisfactorily explained without the dubious assumption of heptacoordination in the iron. The association of haem with cyanide is a two step reaction and there is also some evidence for a two-step association with pyridine. Haem combines with one or two moles of cyanide or alkylisocyanides, but not alkylcyanides [Keilin (118)]. Iron is therefore bound to the carbon atom, not to the nitrogen, and the monocyanide compound with a haem*o*chrome-like spectrum [*cf.* (4, pp. 188 and 39)] has probably the $Fe=C=\ddot{N}^-$ structure suggested by Pauling (12, p. 60) for cyanhaem*o*globin. Carbon monoxide cyanide ferroporphyrins are obtained by the action of carbon monoxide on the dicyanide (not the monocyanide) compounds. Haems in strong sodium hydroxide solution (above 1*N*) form dihydroxyl ferroporphyrins [Keilin (41)], whose absorption spectra resemble those of dicyanide ferroporphyrins, although the former have ionic, the latter covalent, linkages.

Clark *et al.* (42) have investigated the combination of mesohaem with a number of proteins. A variety of proteins forms haem*o*chromes, among them human serum albumin. The latter is surprising, in view of the fact that haemalbumin is not a haem*o*chrome. The results of a spectrophotometric study on methaemalbumin formation are described in a preliminary publication by Rosenfeld & Surgenor (43). Haematin combines with serum albumin in a molar ratio 1:1, while no spectrophotometric evidence for a combination of haematin with other plasma proteins was found.

Haemoglobin

Methods of preparing crystalline human oxyhaemoglobin, haem*o*globin, and carbon monoxide haemoglobin have been described by Drabkin (44), by Kubowitz (45) and by Jope & O'Brien (12, p. 269). Dognon & Gougerot (46) make use of the fact that haem*o*globin does not froth to remove other proteins from it. The crystal form of the haemoglobin of the mule (stallion × ass) is that of the stallion, and that of the mare × ass cross that of the ass (47). Perutz has now published his data on the complete three-dimensional Patterson analysis of horse haem*i*globin crystals (48).

The cobalt complexes able to combine reversibly with oxygen have been reviewed by Michaelis (49). Haurowitz (12, p. 53) has confirmed the old observation of Zeynek that by dehydration of reduced haemoglobin in high vacuum a haem*o*chrome is formed which reverts to haemoglobin on addition of water, and finds that oxyhaemoglobin gives off its oxygen much less rapidly in the dry state than in solution. The dissociation of oxyhaemoglobin is thus to be written: $HbO_2+H_2O \rightleftharpoons Hb.H_2O+O_2$. Pauling (12, p. 57) has drawn further conclusions as to the electronic state of the iron atom in haemoglobin derivatives from their magnetic susceptibilities.

Kinetic and equilibrium data.—New facts have been published which have an important bearing on the haemoglobin-oxygen equilibrium. Roughton

[(12), p. 83, *cf.* (4, p. 268)] has found that Pauling's equation for this equilibrium, which fits the 1929 data of Ferry & Green, fits only the middle part of the curve drawn through the 1931 data of Forbes & Roughton for sheep haemoglobin and the more recent data of Courtice & Douglas (50) for human blood, lying below the points found on the top and bottom of the experimental curve. The experimental points can thus only be fitted by an asymmetric curve. In the derivation of his equation, Pauling had postulated that four indistinguishable haems are arranged as if located at the corners of a square, and that the addition of each oxygen molecule facilitated the addition of oxygen to adjacent but not to diagonal positions. Wyman (5, 12, p. 95) basing his conclusions on the increased symmetry of the equilibrium curve when measured in strong urea solutions (haemoglobin split into two half molecules), on the increased affinity for oxygen and the high interaction energy between haems under these conditions, has suggested that the haems be considered as grouped in two pairs, that intra-pair interaction is stronger than inter-pair interaction, and that stabilising interaction between non-oxygenated haems is more important than the destabilising interaction between oxygenated haems.

Since the equilibrium constants depend on the velocity constants of the "on" and "off" reactions, measurements of the latter would be expected to provide some evidence as to whether haem interaction facilitates or retards the addition of oxygen [*cf.* however, Wyman's remarks (5, p. 481)]. Roughton, Legge & Nicholson (12, p. 67) found that the velocity of combination with carbon monoxide was unaffected if one haem was already occupied by carbon monoxide, and that the initial velocity of oxygen dissociation was unaffected if the reaction commenced with one haem uncombined.

Technical difficulties have so far prevented accurate measurements towards the ends of the "on" and "off" reactions, where there is some evidence that the velocity constants show changes. Roughton, Paul & Longmuir (27) have commenced such measurements, of the equilibrium between sheep haemoglobin and oxygen at very low and very high saturations, where the results may enable the unequivocal calculation of the dissociation constants of the first and the fourth oxygen molecules. Their most interesting finding, further complicating the whole problem, is that the influence of pH, temperature, and dilution on the equilibrium constant for the first oxygen molecule is quite different from the effect of these variables on the partial pressure of oxygen producing half saturation. The methods hitherto used for the adjustment of data obtained under different conditions of pH, for example, to a standard pH will, therefore, distort the data at the lower part of the curve. Johnson & Schlegel (51) find the equilibrium between oxygen and bovine haemoglobin unaffected by pressures up to 680 atm.

Other reactions relevant to the problem of protein-prosthetic group interaction have received further attention. Wyman (5) and Roughton (52) have initiated a discussion on the combination of carbon dioxide with haemoglobin and oxyhaemoglobin. Further data on this problem have been contributed by Kuznetsov (53).

Brooks (54) has further discussed the kinetics of haem*i*globin formation and Legge's interpretation of his results. George (27, p. 365) believes that they can be explained by assuming an easily reducible group in the globin, whereas Lemberg & Legge (4, p. 394) have postulated an easily oxidisable group in the globin. Gibson & Harrison (55) could find no spectroscopic evidence for intermediates between haem*o*globin and haem*i*globin. The war has prevented the publication of studies of Coryell's co-workers on the influence of haem-linked acid groups of haem*i*globin on its reaction with various anions (fluoride, mercaptide, azide, thiocyanate, fulminate), except in the form of theses (56, 57, 58). Apart from throwing new light on the acid dissociation constants of haem*i*globin and its compounds, these studies indicate, but do not yet prove, that there may be a difference with regard to the interaction of their haematins between haem*i*globin produced by autooxidation and haem*i*globin produced by ferricyanide.

Foetal haemoglobins.—Kendrew & Perutz (59) find differences in the crystal structure between foetal and adult sheep haemoglobins. Solubility experiments of Karvonen (12, p. 279) indicate that both foetal and adult sheep haemoglobin are composed of two nearly related components. Dognon & Gougerot (60), by foam separation at different salt concentrations, find two adult forms in the human, while Derrien & Roche (61, 27 p. 368) report that by careful solubility measurements no less than five pigments can be found in the newborn human, two adult and three foetal types, the latter comprising 90 per cent. In the crystals of human foetal and adult haemoglobins, however, Jope & O'Brien (12, p. 269) find no indication for the occurrence of several forms. Fuller & Swarm (62) and Gutfreund (12, p. 197) have found no difference in molecular weight between human adult and foetal haemoglobins in the ultracentrifuge. While adult haemoglobins dissociate on dilution only in concentrated salt solutions, foetal sheep but not foetal human haemoglobin dissociates more readily.

Myohaemoglobin.—Bowen (63) has modified the preparation of myohaemoglobin from horse heart. Large crystals of whale myohaemoglobin have been prepared by Keilin & Schmid (64). Kendrew (65) has published preliminary x-ray data for horse and whale myohaemoglobins [*cf.* (12, p. 149)]. Schmid (66) has studied the electrophoretic properties and the composition of the latter; two to three boundaries are observed between pH 4 and 11 but only one outside this pH range. Papers by Bowen (67), de Duve (68), Rossi-Fanelli & Guilotto (69), and Beznák (70), deal with the spectrophotometric analyses of mixtures of haemoglobin and myohaemoglobin. The last of these publishes figures for the extinction coefficient of the "Soret" band[1] of oxymyo-

[1] The authors follow precedent in their use of the term "Soret band" as applied to porphyrin and haematin compounds, in referring to their characteristic absorption in the violet or near ultraviolet. The Editors do not feel justified at this juncture in arbitrarily imposing alternative phraseology. The term "Soret region" is sometimes used to refer to the violet and near ultraviolet portion of the spectrum, ca. 380 to 440 mμ. Physicists here do not countenance designation of a portion of the spectrum in this manner.—The Editors.

haemoglobin which are much lower than previous workers had found.

Invertebrate haemoglobins.—The haemoglobins of various nematode intestinal parasites have been carefully studied by Davenport (71) and by Rogers (72). These differ from vertebrate haemoglobins by their extremely high oxygen affinity, the low rate of the dissociation of oxyhaemoglobin, and their low carbon monoxide capacity, while their spectroscopic "span" (between the α-bands of oxy- and carboxy-haemoglobin) does not correspond to the low partition coefficient [*cf.* (73, 74)]. Davenport found no evidence for reduction of the perienteric haemoglobin of *Ascaris in vivo*, though it was slowly reduced by dithionite; the body wall haemoglobin was, however, reduced *in vivo*. Rogers could observe oxygenation and desoxygenation *in vitro* and the reactions of some nematode haemoglobins were faster than those of *Ascaris* haemoglobin, but carbon monoxide did not affect the rate of oxygen uptake by the parasites. The great ease with which these haemoglobins are autoxidised to haem*i*globins is also of interest.

Fox (74) has studied oxygen and carbon monoxide affinities of various invertebrate haemoglobins and a chlorocruorin; no general relationship between them was found to exist. Berthier (75) has found two haemoglobins in *Planorbis*, one in the blood, and the other in the nervous system and the muscles of the pharynx and stomach. They differ spectroscopically and in their resistance to alkali.

The relation between the haemoglobin of root nodules and nitrogen fixation remains obscure. Outside the nodules *Rhizobia* do not fix nitrogen in the presence of nodule haemoglobin [Tove & Wilson (76)]. Smith (77) found no evidence that the presence of haemoglobin affected the Q_{O_2} of the nodules; nor was the haematin content of effective and ineffective strains of *Rhizobia* significantly different.

Haematin Enzymes

Cytochromes and cytochrome oxidase.—During an investigation of the haematins extracted from *Corynebact. diphtheriae* by acid acetone, Rawlinson & Hale (78), working in Rimington's laboratory, observed that by distribution between ether and aqueous pyridine-hydrochloric acid, protohaematin could be effectively separated from haematin-*a* which remained in the ether. While the protohaematin is certainly derived from cytochrome-*b*, the haematin-*a* probably constitutes the sum of the prosthetic groups from cytochromes-*a* and cytochrome oxidase. It can be isolated in the same way from heart muscle. Falk, working in Lemberg's laboratory, had approached the problem of the structure of haematin-*a* by studying the properties of a variety of synthetic porphyrins with carbonyl groups in the side chains and their haematins and by comparing them with those of haematin-*a* and porphyrin-*a*. At the First Biochemical Congress, the above workers presented three joint communications (27, pp. 351, 378, 379). The findings of Negelein (79), that haemochrome-*a* shows only a single absorption band at 587 mμ, were confirmed. Porphyrin-*a* differs from all the known porphyrins with formyl or acetyl group side chains, including chlorocruoroporphyrin (one

formyl instead of one vinyl group in protoporphyrin). It resembles most closely oxorhodoporphyrin (2-desethyl-2-acetyl-rhodoporphyrin) in the type of its neutral absorption spectrum and the band of its haemochrome, but differs from it in other respects. Porphyrin-*a* is believed to have one formyl group, vinyl groups (probably two), and an as yet unidentified group responsible for the oxorhodotype spectrum. Analytical figures for the pure compound have not yet been obtained.

Paul, in a communication to the Cambridge Congress, describes the splitting of the sulphur linkage on the prosthetic group in cytochrome-*c* by silver sulphate to obtain ether-extractable haematohaematin, and finds that the reaction may be used for assay of cytochrome-*c* haematin after protohaematin has been first removed by ether and acetic acid. Paul has also found (80) that cytochrome-*c* is not irreversibly changed between pH 1.6 and 12.3. Oxidation-reduction studies by Paul (81) and by Rodkey & Ball (82), which indicate the existence of a haem-linked acidic group with pK 6.8 or 7.7 respectively, may make necessary a reexamination of the structures ascribed by Theorell and Åkesson to ferricytochrome-*c* [*cf.* (2)]. Tsou (27, p. 369) has obtained a modified cytochrome-*c* by digestion with pepsin. Chloroplast cytochromes have been further studied by Hill (27, p. 524). Carruthers (83) reports polarographic studies indicating the presence of -SH or S-S-groups in cytochrome-*c*.

Falk (84, 85) has found that cytochrome-*c* can catalyse the oxidation of ascorbic acid in presence of adrenaline or of certain drugs of the acridine or quinoline series. Its action is that of a nonspecific haematin peroxidase. According to Slater (86), there is no satisfactory evidence for the existence of a separate cytochrome-c_1 with an absorption band at 552 mμ. Slater's factor, which mediates between cytochromes-*b* and -*c* (87), is probably a protohaematin compound which resembles haemoglobin in the ease with which it is destroyed by coupled oxidation with 2,3-dimercaptopropanol, nor does its reduced form possess haemochrome absorption bands. Cytochrome-*b* may be a part of, if not identical with, succinic dehydrogenase (87, 88).

In an investigation of the spores of *B. subtilis*, Keilin & Hartree (89) have shown that although they contain half as much haematin as vegetative forms only a small part is active as respiratory catalyst. Free haematin, but not haemochromes, inhibit succinic oxidase (90), and v. Heyningen (91) found that aerobic spore-forming bacilli, e.g. *B. subtilis*, are inhibited by haematin in high dilution, thus extending earlier observations by Kämmerer (92).

Catalase.—The catalase of *Micrococcus lysodeikticus* has been crystallised by Herbert & Pinsent (93). Its "Kat.-f." (82 to 90,000) is higher than that of human erythrocyte catalase (55 to 62,000). The enzyme contains 1.09 per cent protohaematin and no bile pigment haematin. Its molecular weight is 232,000 and the protein is 85 per cent homogenous in the ultracentrifuge (94). Bacterial catalases have also been studied by Molland (95).

Bonnichsen [(96); see also Chance (97, 98)] has confirmed the earlier results of Lemberg & Legge (99) that some of the haematin groups in horse liver catalase are bile pigment haematin. He finds, however, no bile pigment haematin in human and guinea pig liver catalase, and a smaller percentage

in horse liver catalase, and believes that the bile pigment haematin groups are formed from protohaematin groups during the isolation of the enzyme.

The important studies of Chance have now been reported more fully (97, 98, 100 to 103). Catalase rapidly forms green ("primary") complexes with alkyl hydrogen peroxides with an absorption band at 670 mμ and a Soret band only about half as strong as that of free catalase. All protohaematin groups in the molecule react with RO_2H to form these complexes, which have been formulated as ionic $(Fe^{3+}O_2R)_4$ complexes. It has, however, become doubtful whether the iron linkages are in fact ionic, and it is even possible that a reversible alteration of the porphyrin occurs in their formation (100). Their rather slow, spontaneous decomposition is accelerated by alcohol in a cyclic peroxidative reaction, in which the alcohol used up as hydrogen acceptor is replaced by the alcohol formed from alkyl hydrogen peroxide. The formation and rather slow, spontaneous decomposition of the complexes, as well as their reaction with alcohols, follow the Michaelis theory.

The primary complexes are slowly transformed into the well known red "secondary" complexes with two absorption bands in the green and a Soret band at 425 mμ. They are covalent and react only very slowly with alcohols. The ethyl hydrogen peroxide catalase of Stern is of this type and is not the enzyme-substrate complex in the decomposition of ethyl hydrogen peroxide by catalase (102). The conversion of the primary to the secondary complexes is not a simple monomolecular reaction and its mechanism is not yet clear.

By rapid spectrophotometric measurement of the Soret band, immediately after the addition of hydrogen peroxide, Chance (98) has shown that three protohaematin groups of a 4-haematin catalase and two of a 3-haematin catalase are still able to combine rapidly with cyanide or alkyl hydrogen peroxide irrespective of the concentration of hydrogen peroxide. This primary catalase-H_2O_2 complex thus contains only one of the four haematins combined with hydrogen peroxide, irrespective of the number of intact protohaematin groups (2.7 to 4) in the molecule, the remainder being bile pigment haematins. It is not yet clear whether this represents only a steady state, or a complex of special properties with regard to the reaction with hydrogen peroxide. If a continuous supply of hydrogen peroxide is provided by the notatin-glucose reaction, the primary complex may readily be observed spectroscopically (98, 104). The catalatic reaction is a very rapid one of the primary complex with another molecule of hydrogen peroxide. The Michaelis theory cannot be applied therefore.

These findings also explain why the cyanide inhibition of catalase is "noncompetitive," although it can be demonstrated spectrophotometrically that cyanide in larger concentration than used for the inhibition experiments competes with the hydrogen peroxide bound to the first haematin. The reaction of cyanide with catalase between pH 3.8 and 8.7 is $Fe\cdot OH + HCN \rightarrow Fe\cdot CN + H_2O$ (97, 98)[2], thus resembling the reaction between cytochrome

[2] The expressions FeOH, FeCN of the original articles are here represented Fe·OH, Fe·CN without prejudice as to what convention may finally be accepted.—The Editors.

oxidase and cyanide (105). There is no evidence for haem-haem interaction.

As far as they go, the equations of Sumner (106) describe the reaction of catalase with hydrogen peroxide exactly. What is still in doubt is the structure of the primary complex and the way in which this reacts with the second molecule of hydrogen peroxide. According to Chance, the mechanism of the peroxidative and catalatic activities of catalase are essentially the same; thus the primary alkyl hydrogen peroxide complex is found to react with hydrogen peroxide (103). If the interpretation of this reaction as a direct interaction between the alkyl hydrogen peroxide complex of catalase and hydrogen peroxide ($FeO_2R + H_2O_2 \rightarrow Fe\cdot OH + ROH + O_2$) is correct, the hypotheses of Lemberg (4, p. 443) and of Theorell (107) are disproved. According to the former, the primary hydrogen peroxide complex reacts first with a hydrogen acceptor group in the protein with liberation of oxygen,[3] and according to the latter the reaction consists in an internal electron exchange in a catalase compound with two $Fe\cdot O_2\cdot H$ groups. The kinetics of the haematin enzymes have been discussed by Lu Valle & Goddard [(117), *cf.* also (4, p. 387 f.)] in the light of monovalent intracomplex reaction (Michaelis) without the assumption of radical chains.

George (108), following the reaction between catalase and hydrogen peroxide by manometric methods, observed an initial "burst" of oxygen evolution (α-activity) which, in the course of one or two minutes, falls off to an almost steady state (β-activity). The $\alpha \rightarrow \beta$ change is reversible and thus unrelated to any permanent inactivation of the enzyme. The high initial activity has been confirmed by other workers with the oxydimetric method (109, 110). Chance (104) has suggested that the $\alpha \rightarrow \beta$ change is due to the formation of a secondary hydrogen peroxide-catalase compound similar to those observed with the alkyl hydrogen peroxides, but in the opinion of the reviewers it is difficult to correlate the results of Chance and George. The compound obtained by Lemberg & Foulkes (109, 111), by the action of ascorbic acid on catalase, is a secondary hydrogen peroxide complex. The inhibition of the catalatic activity by ascorbic acid and copper (112) is probably also due to the formation of this complex. The factors which cause the faster transformation of the primary into the secondary complex remain, however, unexplained.

By spectroscopic observations, Lemberg & Foulkes (109, 111) have been led to the view that azide and hydroxylamine are peroxidatively oxidised by the enzyme and that the compounds formed are ferrous NO-compounds of catalase; the oxidation of azide has, however, not been proved directly, and there are still observations which are not explained by the hypothesis.

The very high concentration (1 to 2 per cent) of catalase in *M. lysodeikticus* (93) indicates that the biological function of catalase is not simply the destruction of hydrogen peroxide, thereby strengthening the suggestion put forward by Keilin & Hartree (113) that some other function, probably peroxidative, must be sought for the enzyme. According to Chance (103),

[3] The fact that no *measurable* amount of oxygen is evolved in the peroxidative action of catalase does not invalidate this hypothesis [*cf.* (4, p. 445)].

the essential grouping oxidized in the peroxidative action of catalase is H—C—OH (alcohols; formaldehyde, in the form of dihydroxymethylene; formic acid), H·N·OH [nitrite, *cf.* (114)], and (in the catalatic reaction) HO·OH. The oxidation of carbon monoxide in the intact animal (115) may also proceed via formate and be catalyzed by catalase. On the other hand, Foulkes & Lemberg (116) have found that, at least under certain conditions, catalase protects haemoglobin against irreversible oxidation by hydrogen peroxide.

Chance (97) measures the catalatic activity by following the destruction of hydrogen peroxide spectrophotometrically at 215 mμ.

Peroxidase.—Theorell (2) has recently reviewed the linkage of the haematin to protein. As in catalase, a hydroxyl group is bound to the iron in ionic linkage, whereas in haem*i*globin hydroxide this linkage is covalent.

Fresh data on the kinetics of the peroxidases have appeared in a series of papers by Chance (27, p. 355; 119, 120). Three spectroscopically distinct H_2O_2 complexes (I, II, III) of horseradish peroxidase have been observed. I closely resembles the green primary RO_2H complex of catalase, while II, or III according to Chance (120), corresponds to the red secondary complex of catalase. The bond type in I had previously been considered ionic on the basis of its absorption spectrum, but recent findings of Chance (unpublished) indicate that it is covalent as that in II. Before reacting with the hydrogen donor ("acceptor" of Chance), I is converted into II. The change I→II, while kinetically of monomolecular type, is accelerated by a hydrogen donor or a hydrogen donor group present in the enzyme, which also accelerates the reaction of II with the added hydrogen donor. It is difficult to understand why this should be so, nor is it clear yet why the formation of the secondary complex inhibits the peroxidative action of catalase, but not that of peroxidase. A large excess of RO_2H, not of H_2O_2, partly inactivates the peroxidase and converts it into a green compound with a strong absorption band in the red, probably with oxidation of the porphyrin nucleus. Against the earlier claim of Altschul *et al.*, Chance finds that horseradish peroxidase is able to oxidase cytochrome-*c* quite rapidly (119). Smith *et al.* (121) have developed colorimetric assay procedures for peroxidases and cytochrome oxidase. Ettori (122) has studied the optimal conditions for the purpurogallin method and converted it into a manometric method measuring carbon dioxide output.

Haemoglobin in the Erythrocyte

Dervichian, Fournet & Guinier (123) and Perutz (124) find that the packing of oxyhaemoglobin molecules in the erythrocyte is intermediate between that of a liquid and a solid crystal, an arrangement whereby a high concentration of pigment may be achieved without hindering the diffusion of gases greatly.

Itano & Pauling (125) have drawn attention to earlier reports of birefringence in fully sickled cells, indicating a strong degree of orientation of the haem*o*globin molecules which is not found in the normal erythrocyte. The alteration of the shape of the molecule by conversion of oxyhaemoglobin to

haem*o*globin [*cf.* (126 and 12, p. 269)] and the fact that sickle cell haem*o*globin differs from normal human haemoglobin [Pauling *et al.* (127)] probably explain the hindered rotation of the haem*o*globin in the sickle cell.

Haemiglobin.—The metabolism of the red cell has been dealt with extensively in a review by Granick (128). Due to the difficulties of scientific communications during and after the war, the important papers of Kiese (129, 130) on the reduction of haem*i*globin in the red cell have been overlooked by most reviewers and workers. Kiese's earlier findings demonstrating the importance of the DPN (diphosphopyridine nucleotide) system for the reduction in normal erythrocytes, the importance of the TPN (triphosphopyridine nucleotide) system in the presence of methylene blue, and the role of haem*i*globin reductase, probably a flavoprotein, as carrier between haem*i*globin and TPN, have been fully confirmed. Pennell & Smith (131) make use of the reducing enzyme systems remaining in haemolysates, and of the great stability of haem*o*globin, for the preservation of haemoglobin.

In familial haem*i*globinaemia a deficiency of the reducing enzymes has been shown to exist which is probably due to the lack of flavine mononucleotide or of the apoenzyme [*cf.* (128)]. Fishberg's claim (132) that benzoquinone acetic acid, a haem*i*globin former, is present in the urine in the disease, draws attention to the factor of increased rate of oxidation as well as slower reduction of haemoglobin. Hörlein & Weber (133) report that the haem*i*globin is abnormal and contains a different globin component.

Life span.—Earlier work by a number of methods had established concordant figures for the lifetime of normal red cells in man and dog of about 120 days [*cf.* (4, p. 299; 134)]; Grinstein *et al.* (135) find a somewhat shorter life span in the dog. V. Hevesy (136), Ottesen (137), and Shemin (138) show that in the chick, which has nucleated cells, the life span is 28 days. Ponticorvo, Rittenberg & Bloch (139) find 100 days in the rat, while Niven & Neuberger (140) estimate the life of the rabbit erythrocyte to be 60 to 65 days.

Haemoglobin Catabolism

Verdohaem.—Lemberg & Purdom (27, p. 348) have converted crystalline verdohaemin into biliverdin under conditions which exclude the possibility of removal of a carbon atom from the ring. Their work disproves the earlier claims of Libowitzky (141) and Stier (142) in favour of a carbon-closed ring structure of verdohaem. Biliverdin is able to form a haem-type compound with iron, which is different from the ferrichloride and a haem*o*chrome. It differs from verdohaem, but can readily be obtained from it by alkaline hydrolysis. It is concluded that the ring in verdohaem is closed by a semi-acetal-like oxygen bridge.

Choleglobin, pseudohaemoglobin, and related compounds.—While Liébecq (143) could not obtain conclusive evidence for the reconversion of pseudohaem*o*chrome into protohaem*o*chrome by alkaline dithionite at room temperature, Lemberg & Purdom have now found that cholehaemochrome, pseudohaemoglobin, and also myeloperoxidase, are transformed into haem*o*chrome

with the absorption bands of protohaem*o*chrome by alkaline dithionite at 100°C. While these compounds thus differ from sulphhaemoglobin in their far greater stability to alkaline dithionite, they have all to be considered as containing a carbon-closed ring, in contrast to verdohaem, which does not undergo this reconversion. They should not be called "verdoglobins," since this will lead to confusion with the verdohaem derivatives; the same objection holds against the term "verd" used by Kiese (144) to designate the prosthetic groups of various green haemoglobins. The literature has been reviewed by Lemberg (4, Chapter X) and by Liébecq (145).

Spectroscopically the haem*o*globins or haem*o*chromes of this type are characterized by an absorption band in the red-orange and a low Soret band. Such compounds have also been obtained in the meso series (27, p. 348; 146), together with products of further oxidation which show absorption bands at 660 to 670 mμ in the ferrous state. These can still be reconverted to protohaem*o*chrome, and are intermediates in the conversion of cholehaem to verdohaem. Lemberg & Purdom (27, p. 348) have obtained protein-free green haematins of these two types by exposing haematin or mesohaematin to the action of dithionite and hydrogen peroxide in the presence of cyanide. The cholehaematin (or pseudohaematin) which is obtained by removal of cyanide by dialysis, can be recombined with native globin to a compound spectroscopically indistinguishable from choleglobin, but not giving rise to biliverdin with acids.

The common factor in all these reactions is assumed by Lemberg to be the saturation of a double bond in one of the methene bridges by the addition of hydrogen peroxide, probably in form of two hydroxyl groups, leaving the ring unimpaired but breaking the conjugation of the double bonds in it. In the reaction which leads to the formation of bile pigments, this group is then further oxidised: $>CHOH \rightarrow >CO \rightarrow >O + CO_2$. *In vitro*, however, secondary reactions occur, such as the incorporation of sulphonic acid groups in reactions with dithionite, of $\cdot SCH_2 \cdot CH(NH_2) \cdot CO_2H$ in reactions with cysteine or with the protein part of the molecule, establishing a firm linkage between prosthetic group and protein (as in choleglobin and pseudohaemoglobin) and diminishing the final yield of bile pigment. Finally, side chains too are oxidised in the reaction of haem*i*globin with nitrite and hydrogen peroxide and, after reconversion of the oxidised ring to porphyrin, porphyrins with carbonyl groups in the side chains may be isolated [Kiese (144)].

Bile pigment formation in the erythrocyte.—Foulkes & Lemberg (116) have investigated factors influencing the rate of choleglobin formation. In intact mammalian erythrocytes haemoglobin is oxidised very slowly to choleglobin by hydrogen peroxide generated by ascorbic acid or by the D-amino acid oxidase system, unless the erythrocyte catalase is poisoned; the glucose-notatin system, however, produces choleglobin in the presence of uninhibited catalase, as was found before by Keilin & Hartree (147). Catalase is responsible for a lag in the initiation of the reaction in haemolysates. Destromatisation renders the catalase more sensitive to the inactivation by ascorbic acid plus copper, and increases the rate of choleglobin formation.

Choleglobin formation is catalysed by traces of denatured haemoglobin which may be formed during the process [*cf.* also Valdimirov & Kolotiliva (148)]. Bénard *et al.* (149) have withdrawn their previous claim for the presence of choleglobin in blood.

The presence of bile pigment, probably as bile pigment haematin compound, in normal erythrocytes has been confirmed by Lemberg (150). The negative findings of Gardikas, Kench & Wilkinson (151) were due to the fact that the bulk of the pigment isolated under their conditions was a weakly basic bilipurpurin which is not extracted by the 10 per cent hydrochloric acid they used. György & Rose (27, p. 63) have found evidence for haemolysis by substances able to undergo coupled oxidation with haemoglobin in tocopherol-deficient rats.

Wigglesworth (152) has reviewed the fate of haemoglobin and the formation of bile pigments in insects. Virtanen & Miettinen (153) isolated biliverdin from the green root nodules of the pea after keeping it in the dark. The substance from which this is derived shows an absorption band at 665 mμ and is probably verdohaemoglobin, not choleglobin.

Bile pigment metabolism.—Three groups of workers [London *et al.* (154), Grinstein *et al.* (155), and Gray *et al.* (156, 172)] have now measured the N^{15}-content of stercobilin after feeding labelled glycine. The N^{15}-content of crystalline tetrahydromesobilene-(b) rises immediately after ingestion of the glycine. There is no doubt that 10 to 15 per cent of it in normal individuals, and a much higher percentage in patients with congenital porphyria or untreated pernicious anaemia, cannot be derived from the haemoglobin of erythrocytes breaking down at the termination of their life span. It is not clear yet whether the source of the rapidly formed stercobilin is haemoglobin (e.g., by red cells undergoing immediate destruction on entering circulation, or by bile pigment formation inside the immature red cells of the bone marrow) other haematin compounds (which, different from haemoglobin, are not protected from immediate breakdown, e.g., catalase or myohaemoglobin), free haematin (which may be formed in excess of globin and decomposed in the developing erythrocyte), or an independent synthesis.

Legge (157) found sulphanilamide to increase the excretion of stercobilin and to increase markedly the ratio mesobilene-(b) to tetrahydromesobilene-(b).

A comparison of the amount of bilirubin formed daily in man and dog with that calculated from haemoglobin breakdown shows that bilirubin is certainly the major product [*cf.* (4, p. 509)]. Normally other pigments, such as the dipyrroles, cannot therefore be the major primary products of haemoglobin catabolism. This holds even if some bilirubin should be formed by independent synthesis, for which there is so far no evidence. The claims of Bingold (38) that pentdyopent should be considered the major product of haemoglobin catabolism, and that the bile pigments constitute only 10 per cent are not justified.

It is known, however, that the stercobilin excretion is lower and varies between 40 to 70 per cent of that calculated from haemoglobin breakdown.

Siedel, Stich & Eisenreich (35) have shown that mesobilifuscin and promesobilifuscin of the faeces may arise by oxidation-reduction of bilirubin in the intestine. The formation of the dipyrroles thus explains the differences between bilirubin and urobilin formation.

Biosynthesis of Porphyrins and Haematin Compounds

The demonstration, by means of glycine labelled with N^{15}, that the synthesis of the porphyrin nucleus and of haemoglobin proceeds in the avian erythrocyte *in vitro* [Shemin, London & Rittenberg (158)], has opened a new field of reseach. This synthesis also occurs in sickle cell blood (159) and in reticulocytes of rabbit and man, though not invariably in the latter. Shemin (138) has reviewed the recent progress in this field. The rate of haemoglobin production revealed by the isotope technique is rapid and is further increased in anaemias caused by bleeding or phenylhydrazine, in sickle cell anaemia and in polycythaemia, but is only about 75 per cent of the normal in untreated pernicious anaemia [London *et al.* (134), Altman *et al.* (160), Niven & Neuberger (140)]. N^{15} from serine is rapidly incorporated in the haem of haemoglobin via glycine (138). Tryptophane [Schayer *et al.* (161)] or aspartic acid [Wu & Rittenberg (162)] are not direct precursors for haem synthesis.

The N^{15} of glycine is incorporated to the same extent in the two acidic and the two nonacidic pyrrole rings of haem, which makes their derivation from a common precursor pyrrole likely [Muir & Neuberger (163), Wittenberg & Shemin (164)]. Using $H_2NC^{14}H_2CO_2H$, Altman *et al.* (160) have shown that the α-CH_2 group is incorporated in haem, whereas the carboxyl group of $H_2NCH_2C^{14}O_2H$ is not [Grinstein *et al.* (135)]; these findings have been confirmed by the Columbia University workers (138, 165). Nevertheless glycine contributes two carbon atoms, not one, per pyrrole ring [Radin, Shemin & Rittenberg (165), Muir & Neuberger (166)]. One carbon atom forms an α-carbon of the pyrrole ring, whereas the second carbon (derived from another molecule of glycine whose nitrogen is not used) yields one methene bridge. This is supported by the formation of $C^{14}O_2$ in the chromic acid oxidation of mesoporphyrin, obtained from C^{14}-haemin, by which the pyrrole rings are transformed into methylethylmaleimide and haematinic acid without loss of carbon.

The remaining carbon atoms of the haem stem, at least partly, from acetic acid. Using deuterio-acetate, with deuterium in the methyl group, Ponticorvo *et al.* (139) have shown that a third of the hydrogen atoms of haem are derived from the methyl group of acetate. Haem formed from $C^{14}H_3CO_2H$ is about six times as radioactive as haem formed from $CH_3C^{14}O_2H$; the latter contains about half of the activity in the side chain carboxyls, but some also in the nonacidic pyrrole rings; $CH_3C^{14}O\ CO_2H$ is also incorporated, not, however, $CH_3C^{14}OCH_3$ or $C^{14}O_2$ (138, 165). Bufton, Bentley & Rimington (167) found no incorporation of $C^{13}O_2$ or $HC^{13}O_2Na$ in the haemin of surviving chicken erythrocytes, whereas Armstrong *et al.* (168) had found some radioactivity in the haemin of a rat after implantation of a pellet of $CaC^{14}O_3$.

Rimington concludes from his experiments that no compound involved in the citric acid cycle can be a precursor in porphyrin synthesis.

It does not appear likely that the complex molecule of protoporphyrin should be directly synthesised from glycine and acetic acid by an enzyme fitting many small pieces together in the correct position. There is a 20-fold dilution of deuterium in the synthesis of deuteriohaemin from deuterio-acetate (139). Legge & Lemberg (4, pp. 639, 640) have suggested that a condensation of two molecules of α ketoglutarate or a related substance with glycine may lead to a precursor with the side chains of uroporphyrin (acetic and propionic acid groups). They have drawn attention to the pyrrolidone tricarboxylic acid isolated from liver extract by Dakin & West (169) which probably contains an acetic acid group in one α-position and acetic and propionic acid groups in the β-positions. A similar pyrrole or pyrrolidine compound with an acetic acid group in one α-position (possibly derived from the second molecule of glycine and a carboxyl group (derived from glycine) in the other, would be a suitable precursor. In the condensation to the porphyrin ring the α-acetic acid side chain would supply the methene bridge (all four of type I porphyrins and three of type III porphyrins). Both of the carboxyls derived from glycine would be eliminated, as well as the carboxyl proximal to the carbonyl group of α-ketoglutaric acid. Since there is not necessarily an exchange of C^{14} between the two carboxyls of α-ketoglutaric acid [Potter & Heidelberger (170)], $C^{14}O_2$ might well be taken up by fixation into α-ketoglutaric acid, but lost again in the condensation. Rimington's objections against such a precursor are, therefore, inconclusive.

The assumption of a decarboxylation of the porphyrins, or rather of their pyrrole precursors, is now better supported than the earlier assumption of a "detoxifying" carboxylation, at least for the relation between coproporphyrin and protoporphyrin. A dog which had been transfused with blood of another dog containing haemoglobin tagged with N^{15}, and in which haemolysis had then been produced by phenylhydrazine, excreted protoporphyrin containing N^{15}, but coproporphyrin without N^{15} [Grinstein *et al.* (171)]. The incorporation of N^{15} in urinary and faecal coproporphyrin and uroporphyrin in congenital porphyria is more rapid and leads to higher isotope concentrations than its incorporation in haem [Grinstein *et al.* (155), London *et al.* (27, p. 357), Gray & Neuberger (172)], although in *Corynebact. diphtheriae* Rimington (12, p. 241) found the N^{15}-content of its coproporphyrin lower than that of the intracellular haematins.

The data of Grinstein *et al.* (155) and of Gray & Neuberger (172) support the assumption that coproporphyrin formation precedes uroporphyrin formation (as well as protoporphyrin formation). A pathological lack of a normal decarboxylation process in porphyria appears to the reviewers, however, a more likely explanation of the uroporphyrin formation in this disease than a pathological carboxylation process (4, p. 637 f.), and a precursor with the side chains of uroporphyrin a more likely primary precursor than one with those of coproporphyrin. Glycine-N^{15} is also rapidly incorporated in stercobilin (*cf.* above) and in the tripyrrylmethene prodigiosine from *Serratia*

marcescens [Hubbard & Rimington (173)]. Benzoate and cyanide reduce porphyrin excretion in rats, while glycine and pteroylglutamic acid increase it (174, 175, 176).

Little is still known about the rate of synthesis of other haematin compounds. The incorporation of radioactive iron in myohaemoglobin is slow [Theorell (Communication to Cambridge Congress), Dreyfus & Schapira (27, p. 130), *cf.* also (177, 178)]. The same appears to hold for cytochrome-*c* (Theorell), although Beinert & Maier-Leibnitz (179) could label cytochrome-*c* in heart and skeletal muscles of young iron-depleted rats by injection of Fe^{55}. Although there is no evidence for the passage of cytochrome-*c* injected intravenously into the cells of organs (180, 181, 182), and although the cytochrome-*c* in muscles is not decreased but rather increased in lead anaemia [Vannotti (12, p. 253)], stringent proof is still missing that the increase of cytochrome-*c* in the regenerating liver [Drabkin (12, p. 35)] is due to its synthesis in this organ. Thyroxine increases the cytochrome-*c* in rat tissues, but lowers its increase in the regenerating liver (183). Theorell found that radioactive iron is incorporated in the erythrocyte catalase of the guinea pig as rapidly as in its haemoglobin, while it is taken up still more rapidly in liver catalase. Since the catalase haemin was isolated and no bile pigment haematin was found in guinea pig liver catalase, the rapid iron incorporation in the liver catalase can hardly be due to an exchange of bile pigment iron with ionic iron.

Granick has shown that the biogenesis of chlorophyll is closely linked with that of porphyrins. Some mutants of *Chlorella vulgaris* contained the magnesium complex of protoporphyrin (184), others that of a phaeoporphyrin-*a* (personal communication). The chlorophyll-*c* found in some algae resembles the latter and is a magnesium complex of a phaeoporphyrin-*a* without the phytol ester group present in protochlorophyll (185). These compounds thus represent stages between protoporphyrin and chlorophyll.

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IMMUNOCHEMISTRY

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A simple enumeration of the work in this field since 1946, and the relevant bibliography, would occupy more space than is at the disposal of the author. He is therefore obliged to omit completely or partially a certain number of important problems, i.e. the blood group substances, phenomena of anaphylaxis, and complement, although outstanding advances have been realized in these domains, especially as regards their quantitative aspects. The reader is asked to refer to recent reviews by other workers i.e., on immunochemistry in general (1 to 4), on the bood group substances (5), on the chemistry of immunopolysaccharides (6, 7), on the cellular theory of immunity (8), on the mechanism of defense (9), on the mechanism of immune reactions *in vivo* (10), on infection and immunity (11), on the immunochemical aspects of allergy (12), on the unitarian hypothesis of hypersensitivity and immunity (13a), on the nature of the bacterial surface (13b), on the immunological research carried out in Germany between 1939 and 1946 (14), on the relationship between the processes of fertilization and those of immunity (15), on immunity against parasites (16), and on the phenomena of immunity in lower vertebrates and invertebrates (17).

Four volumes on immunology by Doerr have appeared recently, two of which are devoted to antibodies, one to antigens, and one to complement. This work ,compiled by an author who has been active in immunology almost since its beginning, is particularly interesting because it subjects various new findings to extensive criticism (18). A treatise on immunity in Italian has been published by Carlinfanti (19). The reviewer has written a monograph on the serum globulins where he develops his hypothesis as regards their biological role and their relationship to the function of antibodies (20). Kabat & Mayer discussed the general development of quantitative immunochemistry and have prepared a manual of immunochemical techniques which will certainly prove of great value to all workers in this field (21).

The IVth International Congress of Microbiology at Copenhagen in 1947 (22) and the First International Congress of Biochemistry at Cambridge in 1949 (23) permitted the immunochemists of many countries to discuss their various problems. Unfortunately, the very interesting exchanges of ideas during discussions at Cambridge will never be published.

Certain fundamental aspects of immunochemistry have been investigated in great detail while others seem to remain in obscurity. For example, publications on the fate of antigen-antibody complexes when antigen is introduced into an immunized organism, are very rare. Very often the progress of our knowledge is due to the introduction of a new technique, and it would

seem that such problems might be resolved by the use of radioactive tracers. Two examples of the use of isotopes in immunochemistry are (*a*) that it is possible to establish that labelled antibody against organs lodges in the tissue of which the constituents were used for the immunization (24), and (*b*) that a labelled antigen is accumulated in the lung of a sensitized guinea pig (25). But it would also be interesting to know what becomes of the antigen-antibody complexes thus formed. Are they eliminated or degraded and, in the latter case, does the union with the antibody facilitate the degradation of the antigen? In other words, has the antibody a passive role of "fixer" or of "carrier" for the antigen, or does it have an active role, for example, as a specific apoenzyme which, with the co-operation of a nonspecific cellular coenzyme, would degrade the antigen?

Antibodies

The statistical treatment of numerous essays of various antibody titers led Carlinfanti (26) to postulate that there exists a predisposition for the immunization and that this property is hereditary, but not dominant. However, for two different antibodies in a given individual, the titers are not necessarily parallel, but for a population there is a statistical parallelism in the antibody response. Stegel found a poor antibody response in mongoloids immunized against TABT. These antibodies disappeared rapidly from the circulation, although the γ-globulin composition of the serum appeared normal. He postulates that this peculiar response is due to a hormonal deficiency in this population (27).

The importance of nutrition, vitamins, etc. in hematopoiesis has been the object of several reviews, of which the most recent was published in France by Aschkenasy (28). It was already known that newborn mammals are difficult to immunize. The same difficulty has been demonstrated with chicks, which gave very weak precipitin titers of antibody to beef serum up to the age of four weeks (29).

As is known, the production of antitoxins in hyperimmunized horses continues during months after the last injection of antigen. A relatively high titer of diphtheria antitoxin was still found 13 months after the last injection in a horse that had been repeatedly immunized and had furnished serum for five years (30). But the ability of the horse to produce antitoxin, after having already served as a producer of another antiserum, is reduced (31). When one injects the antigen with adjuvants into the horse, there is an augmentation of euglobulin unrelated to the antitoxin (31). New details have been published by Freund *et al.* on the use of commerical emulsifiers and dead mycobacteria as adjuvants. This technique can provoke in the guinea pig a hypersensitivity to picryl chloride and other substances (32). The replacement of tapioca by glycerin as adjuvant for tetanus immunization gave similar antitoxin production without provoking excessive lesions (33).

In general most workers have taken into consideration only the antibodies which appear in the blood (humoral immunity) of the immunized animal.

Now, numerous studies have appeared attempting to determine antibodies in tissues or even in the different cells. These studies have as their goal the delineation either of the place of formation of different antibodies which appear afterwards in the circulation, or the demonstration of the presence of tissue antibodies (local immunity of Besredka) which appear in the serum only in the later stages or not at all, e.g., the case of the virus of lymphogranulomatosis inguinale in the mice studied in this laboratory [(34a); *cf.* the review by Lepine (34b)]. The recent work of Schlesinger (35) confirms these results with equine encephalomyelitis virus. Several authors confirm the local presence of antibodies to trichomonas in the vaginal mucous of the cow (36). Biro finds the agglutinins and Wasserman antibodies in the bone marrow of patients more often and sooner after infection than in the serum (37).

It is evident that the path of introduction of the antigen (or its localization, or tissue affinity) can determine the place of appearance of the antibody. Examples of these facts are numerous. Here, however, are some recent studies. The intravenous injection of poliomyelitis virus in the monkey confers only a negligible immunity, in contrast to the intramuscular injection, while the infection provokes a strong concentration of antibody in the anterior horn of the spinal cord (38). On the other hand, the appearance of antibodies in the blood is more rapid when one administers the virus to the monkey orally than it is by intracerebral injection (39). The intravenous injection in rabbits of erythrocytes or of bacterial suspensions permits a high titre of antibodies to appear in the spleen, while such is not the case after subcutaneous injections. The thymus does not seem to participate in the formation of antibodies (40). Fragments of spleen from rats which have been immunized previously with sheep erythrocytes liberate antibodies *in vitro* (in the presence of oxygen and carbon dioxide, and none in the presence of nitrogen alone), while fragments of the mesenteric glands and of the thymus liberate no antibody at all. When the immunization has been made by the intraperitoneal route the reverse happens (41). For the preparation of precipitating rabbit antisera Vink (42) obtained the best results by injecting intramusculary alum-precipitated proteins. In the chicken, the injection of a protein intraperitoneally, in contrast to the intravenous route, did not result in good antibody response (43). In unpublished preliminary experiments in this laboratory, Bussard observed differences in the quantity and quality (precipitating and nonprecipitating) of antibodies against chorionic gonadotrophin, depending upon whether the rabbits were splenectomized or not and upon the route, intravenous or subcutaneous, of the injections. The results obtained by Heidelberger *et al.*, after injecting protein antigens into the horse by different routes, have not been confirmed using diphtheria toxoid as antigen; resulting antitoxins had the same properties (44). In contrast, Perez, who immunized horses to the pig pseudoglobulins, observed at the start of the intravenous immunization the existence of several precipitant systems and perhaps of nonprecipitating antibodies, while after pro-

longation of the immunization, as well as after subcutaneous injections, the reactions were of the antitoxin flocculation type and the antibodies were in the T (or γ_1) fraction in electrophoresis (45).

A monograph on oral, intranasal, intraconjunctival and intracutaneous immunization has been published recently (46). This observation may be added, that the ingestion of dead Brucella by humans provoked the formation of agglutinins, but no skin sensitivity (47).

Once again an attempt to produce agglutinins *in vitro* by suspensions of cells (leukocytes, macrophages, lymphocytes, spleen cells, bone marrow of the rat) failed (48). Since the probability exists that at least one of these kinds of cells actually synthesizes the antibody, the failure of the above type of experiment suggests that the mechanism of antibody formation takes place in at least two stages, the last being the synthesis of antibody.

In spite of the effort made in the course of years by numerous research workers to specify the type of cell capable of synthesizing globulins, the controversy continues. The disagreement is so striking that one finally asks oneself if it is not a matter of different definition given to the same kinds of cells. In a general review, White (49) cites numerous works and gives his point of view, that lymphocytes contain antibodies, but perhaps do not manufacture them.

Estimations of nucleic acids in the lymph nodes have been made by two American research groups with differing conclusions (50, 51). Both established that desoxyribonucleic acid does not increase in the nodule, whereas there is a sizeable increase of ribonucleic acid. This increase coincides quite well with the maximum production of antibodies and cellular proliferation. By one group, the cells synthesizing the antibody are said to be the progeny of lymphocytes (51); by the other group they are plasmocytes (50). Thus Ehrich *et al.* agrees with the Scandinavian workers (Bing, Gormesen, etc.).[1] Fagraeus studied the morphology of fragments of rabbit spleen during incubation and the formation of antibodies after a recall injection (53). She claims now that the antibodies are formed during the development of nondifferentiated reticuloendothelial cells, which can develop later into plasmocytes. The liberation of antibodies during the incubation of tissues of rats and mice has been already cited (41). Thus, we have come to believe that there might be a secretion of antibodies by certain cells and just not a liberation of antibodies by their lysis. This would explain perhaps the relatively feeble amount of antibody in the extracts of lymphatic nodules, and the negative results of Habel *et al.* (54) who tried to reproduce the experiments of Harris *et al.*, except that they washed the lymphocytes in order to eliminate the remains of the serum or of the lymph. Habel points out that the total quantities worked with are so small that the slightest errors influence greatly the results in experiments of this type. In experiments still not completed, the reviewer observed that extracts of mobile well-washed cells (about 95

[1] For Godlowski (52), the eosinophiles would contain the anaphylactogen protein in the sensitized guinea pig.

per cent lymphocytes) of the different lymphatic tissues of the normal horse have a very low total yield of globulins. Analogous extracts, but coming from human tonsils, from thymus of calves (55), and from popliteal glands of normal or immunized rabbits (as well as the lymph of the same animals) have been studied by chemical and physical methods (56). These investigations revealed that the extracts contain, in addition to albumin and nucleic acid, much protein corresponding in mobility to α- and β-globulins. The proteins which correspond in mobility to the γ-globulins are very low in concentration and their sedimentation constants are not similar to that of γ-globulin. The glands of the injected animals contain more total protein but no more γ-globulins. One would therefore be tempted to conclude that the lymphatic glands do not store antibodies. However, it must not be forgotten that, even for a rather high titer of agglutinin, the actual quantity of antibody may be much below the sensitivity of present physical methods; and it is also possible that antibody precursors exist in the cell with electrophoretic mobilities and/or molecular weights different from those of the usual γ-globulins (56).

The rôle in the formation or liberation of antibody by the corticotrophic and adrenocortical hormones, described by White & Dougherty (49), is very much in debate. The increase of antibody found by these authors was at least very slight (differences of one tube in an agglutination test). By certain authors no effect of the hormones could be found (57). Others observed an increase of antibody or of globulins: Arocha & de Venanzi experimented on dogs after hepatectomy or splenectomy and they attributed the increase in serum globulins to a discharge of protein by the spleen (58). Simmonet, using rats, found an increase of globulins after the injection of the above hormones but also after administration of other organ extracts (59). Murphy & Sturm demonstrated a greater response in adrenalectomized rabbits than in the controls, even when the former received injections of hormones to prevent the hypertrophy of lymphoid tissue (60). Very clear results were obtained by Bisset. His earlier research showed that amphibians and fish produce antibodies when they are maintained at 20° C., but that at 8° C. no detectable antibody was produced. However, frogs maintained at 8°C. and receiving cortical hormone behaved in respect to the serum agglutinins much like the controls at 20° C. (61). Experiments made in this laboratory (62) show that the injection of adrenocortical extracts into rats, immunized intravenously against *Salmonella enteritidis* and sheep erythrocytes, provoked at the same time an important blood lymphopenia and involution of the lymphoid organs with a significant increase of antibodies. However, histological examination of the spleen showed at the same time a decrease of the surface of the perifollicular collar in the white pulp, and a proliferation of transitional cells.[2] It therefore appears difficult to decide in favor of the one or the other of the two hypotheses cited above on the origin of antibody, much more so because the animal species, the dose of hormone, and the type of experiments, i.e., first immunization, recall dose, antigen, route of injection,

[2] For further discussion and more bibliography, *see* (62).

etc., have often been different. Likewise, the results of experiments on the action of x-rays on the formation of antibody are difficult to compare (57 c, 63). Acetylcholine is said to stimulate the production of globulin, while eserine, strophanthin (64), and histamine (59) do not have any action.

The study of the transfer of antibody from the mother to the infant has been actively investigated. In man and rodents this transfer can take place, before the formation of the placenta, through the layers of the vitellin sac and after birth, as in other animals, by the feeding of colostrum (65). Detailed data have been collected comparing the concentrations of various proteins in the serum of the mother and in the umbilical blood of premature babies (66). In addition, studies have been made on the composition of colostrum of cows (67, 68), and on the passage via colostrum of different antibodies in calves (67, 68, 69), lambs [McCarthy & McDougall (23, p. 25)], and in foals (70). The passage into the offspring is made in less than 45 min. (70). but does not occur at all, or very little, more than 24 to 48 hr. after birth (23 p. 25, 67, 69). The feeding of antibodies in milk of hyperimmunized mares or of antiserum, even mixed with the milk of the mother, increased only very little the circulating antibodies in the young (69, 70). The pre-colostrum is, according to Blakemore, 20 times richer in antibody than the serum (22, p. 310). The explanation that the absorption of globulins of the colostrum occurs as a result of deficiency of digestive proteases finds some support in the observation of Werner (71) that such a deficiency exists in the prematurely born. But the weak absorption of the globulins of the serum administered by feeding seems to contradict this explanation. Opposition is also found in the experiments of Dent & Schilling (72) in which, in dogs, homologous serum administered by mouth was absorbed intact, without digestion, while other proteins were absorbed only after having undergone extensive degradation. Furthermore, Burrows *et al.*, continuing their studies on anti-cholera immunity, established in man and guinea pig that the antibodies can easily cross the intestinal wall in both directions and can even be excreted in the feces in high concentration (73). The active immunity of chickens towards infectious bronchitis can be transmitted passively through the egg to the chick by the intermediary of the vitellin sac (74).

Several authors reported upon the passage of different antibodies of serum into the cerebrospinal fluid (75), into the nasal secretions (76), and into different parts of the eye (77). Medawar (78) studied the role of blood and of lymph in the destruction of skin-homografts transplanted into various tissues.

Fractionation of horse serum precipitated by dialysis at different pH's, gave three fractions of globulins having different isoelectric points (79), each fraction being said to correspond to antibody with a different function: agglutinins, hemolytic amboceptor, antitoxins (80). Human sera possess anallogous fractions (80). Many human antibodies are found in globulins precipitable by carbon dioxide (81).

Purified rabbit-antiprotein antibodies have been obtained by acid dis-

sociation of specific precipitates of diazo derivatives of antigens (82). Purified solutions of antibody were proved to be homogeneous in electrophoresis and in ultracentrifugation (83 a). Deutsch *et al.*, have presented useful techniques for the isolation of γ-globulins in many species (83 b). Subfractions of fractions II and III, prepared by the ethanol method, permitted the purification of various human antibodies (84). A study of anomalies in electrophoretic diagrams of human sera was made by Hoch (85). Electrophoretic analysis of γ-globulins separated by electrophoresis shows their heterogeneity (86). Hemagglutinins and typhoid agglutinin [for quantitative estimation *see* (87 b)] appear in the γ_1-globulin (isoelectric at pH = 5.8) whereas diphtheria antitoxin of human sera is found in γ_2-globulin with an isoelectric point around pH 7.4 (88). The human γ-globulins have a molecular length of 230 Å, apparently uniform at least in dilute solutions (89). Ten per cent of the proteins of cow whey correspond to lactoglobulins which contain antibodies. They are either eu- or pseudoglobulins. The principal constituent (75 to 92 per cent) has a molecular weight of 180,000 (90). The amino acid composition of lactoglobulin is very similar to that of other immunoglobulins (91). According to Porter (23, p. 142) rabbit antibodies have a single peptide chain with a terminal grouping of alanyl-leucyl-valyl-aspartic acid. By controlled papain digestion he obtained fractions which, without precipitating the antigen, inhibited its flocculation by the complete antibody.

The lyophilization of human sera lessens their titer (92). The heating to 67 to 70° of horse antihemocyanin serum suppresses its flocculating properties, but not its power to combine with the antigen (93). This was true also of human antidiphtheria serum heated to 56°. The product which reacted with the antitoxin was not serum albumin, but was in the crude serum albumin faction. Electrophoresis of the heated product did not reveal the formation of any complexes (88). The denaturation of antitoxins by urea follows a first order reaction in the beginning, but afterwards is more complicated (94). A concentration of one part of ozone in 1,000 of oxygen destroys the isoagglutinins (95), periodic acid destroys the horse antipneumococcal antibody (96), while treatment with alkali destroys the complement-fixing capacity of an antibody-antigen system (97).

The existence of different diphtheria and tetanus antitoxins in the serum of hyperimmunized horses has been confirmed by electrophoresis, by fractionation (98, 99) and by fixation of the diphtheria toxin on hemolysin (100 a). The same heterogeneity has been observed with horse antipneumococcus antibodies (differences in solubility, in protective power and in precipitation by rabbit anti-horse serum). One of the fractions could be crystallized (100 b). A method of extracting antitoxin from blood clots has been described (101), as well as several practical techniques of purification of antitoxins (102 to 105) and of antivenoms (106).

The presence in the serum of syphilitics of spirochetocidal antibody (107) and of antibody capable of immobilizing the spirochetes in the presence of complement, differing from the reagin, has been demonstrated (108). The

reagin seems only to be a "carrier" (see below) the amount of which is abnormally increased in syphilitics. According to Neurath *et al.* (109) there exists in normal serum a substance capable of inhibiting "false positive" reactions. The reviewer feels that this can be interpreted as being due to syphilitic reagins possessing other specificities. This does not contradict the hypothesis of the "carriers," since for lipids a certain degree of specificity with numerous possibilities of cross-reactions can exist (see below) (150).

A detailed study of antibody in foot-and-mouth disease has been published by Brooksby (110). The concentration of antibody in the γ-globulins in the sera of normal pigs and cows has been studied by Cameron (111) and the electrophoresis of antiviral sera of the rabbit and the chicken by Koprowsky *et al.* (112). The antiprotein antibodies are in the γ_1-globulin in chickens, but an important part of the α-globulins co-precipitates with the specific precipitation (43).

In human serum, there exists a nonspecific inhibitor of hyaluronidase, similar to the albumins, and specific inhibitors, antibodies, which are globulins (113). Antistaphylocoagulase might also be an antibody (114), but seems to be part of the α- or β-globulins (115). In certain pathological states one notices cases with increase of the α-globulins [lymphogranuloma (116), articular rheumatism (117)] or of β, followed by α-globulins [in myocardial infarctus (118)]. It is probable that one can parallel these with the observations of Löfström who has been able to reproduce in the rabbit the appearance, in 24 hr., of substances provoking a nonspecific swelling of the pneumococcal capsule; he thinks that this appearance is a result of tissue destruction (22, p. 320; 119).

By definition, an antibody is characterized by its specific reaction with antigen. It follows that to prove the existence of an antibody a specific reaction must be demonstrated. But such a reaction is not necessarily easily visible, as is precipitation, which served many authors as the only plausible criterion of the presence of antibody or of the antigenicity of a substance (18). It seems logical, however, that a new method which would allow us to demonstrate a reaction, provided that it be specific, would substantiate the claim for the existence of an antibody. Further on the question of specificity will be discussed. For a few years, several cases have been presented in which indirect methods have permitted the demonstration of antibodies that are designated as "univalent," "blocking," "nonprecipitating," or "inhibiting." Several publications are concerned with the evidence for, and the properties of, such antibodies, as well as of a third type, "cryptoagglutinoids," of the blood groups (120). The phenomenon of prozone, described for the incomplete anti-Rh antibodies (121), has been explained in the case of agglutination of bacteria, as due to the existence of incomplete antibodies (122).

The study of precipitating sera has shown that a part of the antibodies can precipitate only in the presence of easily precipitable antibodies (123), and that at the start of the immunization, one finds only nonprecipitating antibodies (124). In rabbit antichorionic gonadotrophin sera, the presence of

precipitating and nonprecipitating antibodies has been demonstrated (125). Quantitative methods have been applied to the study of blocking antibodies in the sera of rabbits immunized against pollen (126). The thermolability of sensitizing antibodies has been confirmed and the rôle of the thermostable antibodies in sensitization has been discussed (127).

It seems that sufficient indications exist to warrant further investigations into the possibility of the existence of auto-antibody. It deserves much more attention, as it would explain numerous pathological states. We now know that normal constituents of an animal, after fixing small foreign molecules (toxic gas, dyes, etc., see below), provoke the formation of antibody. It remains to be proved that at least, in certain cases, these antibodies are capable of reacting, by a kind of cross-reaction, with the normal constituents. However, new cases of true auto- or iso-antibodies have been shown recently. The serum of the adder specifically neutralizes its own venom (128); the injection of an extract of the comb of the cock into cocks gives their sera the power of neutralizing the effect of the male hormone on the growth of the comb, as does rabbit antiserum to the same comb extract (129). Actin, extracted from the muscle of the rat, is antigenic for the rat (130). Utilizing his technique of agglutination of collodion particles which had adsorbed antigen, Cavelti (131) continued his research on the auto-antibody in pathological conditions (glomerulonephritis and rheumatic fever). These results have been confirmed by Lange *et al.* (132), but contradicted by other workers (130). Antibodies are not found (134 d) in the serum of a monkey, in which one provokes encephalomyelitis by injection of brain homogenate with adjuvants (134 a, b, c, d). However, the hypothesis of their existence would explain the experimental facts and they may not be found because the antibody is fixed in tissues. These experiments have been confirmed in France on monkeys and guinea pigs (309). The substance which provokes this encephalomyelitis has been found in man, monkey, rabbit, and chicken, but not in the frog or the carp; it withstands boiling, formalin, and ultrasonic treatment (134 b).

If the reader feels that the author has devoted too much space to the questions raised by the above discussed recent studies on antibody, it is that he has the impression that the problem is badly stated. Actually, one generally considers the formation of antibodies, as a "defense mechanism." But, without speaking of the unfortunate effect that this mechanism can provoke (anaphylaxis), it seems useless to envisage a special mechanism for the synthesis of antibody and to place it in opposition with that of the globulins called "normal." No known property, with the exception of the given specificity under consideration, distinguishes the antibodies from the globulins of an animal that we consider as normal, because it has not been sick, or in the hands of an immunologist. It seems, on the contrary, that all their chemical, physical, immunological (as antigen), and physiological (place and method of formation) properties are identical [cf. also Haurowitz *et al.* (123)]. The idea of equating globulins and antibodies appears natural enough. It has

been expressed independently by Boyd and the author during the war, but whereas Boyd in his book distinguishes "carrier globulins" and the antibodies, the author equates them, the rôle of the antibodies being to "fix" and probably to "carry" the antigen. It is evidently difficult enough to prove experimentally the hypothesis that the mechanism of formation of antibodies is not a special defense mechanism, but a normal physiological mechanism inherent in the formation of globulins. The author has been able to accumulate a certain number of arguments in favor of this hypothesis (20, 23 p. 446). We will mention here only the question of specificity which is the most characteristic property of the antibody and we would like to recall that on the one hand this specificity is not very strict in many immune reactions, and, on the other hand, that there are cases when a certain degree of specificity is shown in the case of "carriers." If one considers the chemical composition of the principal groups of biological substances, one can predict that the possibility of the existence of variation in antigenic configuration should increase from lipids to carbohydrates to proteins. One does not know true "carriers" of proteins in normal serum, but reactions of precipitation between extracts of organs and the serum of the same animal have been obtained. The serum contains protein-protein complexes and in numerous pathological cases one finds profuse changes in the electrophoretic patterns when there is tissue destruction and often reactions between the serum of the sick and extracts of the injured organ. With the carbohydrates one can point to numerous cases of immunological cross-reactions. There are, on the other hand, the reactions which normal serum gives with certain polysaccharides (agar, certain vegetable gums), and if the reaction of normal sera with glycogen (23, p. 446) is demonstrated to be specific, one could then speak of a "carrier" of glycogen. Finally, among the lipids one knows only little or nothing of really specific immunological reactions [see, however, the precipitation of phospholipids by antilipovitellin antibody; Banks, Francis, Mulligan & Wormall (23 p. 467). Cf. also (150)]. Kahn showed that in refining his serological test, one finally had reactions with normal sera (22, p. 345) and facts showing the existence of a certain degree of specificity among carriers of lipid have been cited (20). Thus, it would seem that even the property of uniting by specific reaction is not a special characteristic of the antibodies. Let us say finally that if it were clearly demonstrated that the flooding of an organism with small molecules can provoke the formation of specific globulins, it is not improbable that with very fine methods one could show the existence of proteins in all normal sera that can unite specifically with products of normal metabolism.

Antigens

Antigenicity.—The publications of Loiseleur (135) seem to pose the problem of antigenicity of small molecules again. We must however be prudent and wait until the conclusions are confirmed before admitting them. Here is the résumé of his experiments. By injecting rabbits at very short intervals

with large quantities of simple organic molecules (i.e. ethanol, sugars, amino acids, phenol, etc.) Loiseleur establishes that the viscosity of the sera of these animals increases when one adds to them the injected substance. Viscosity has already been shown to change during antibody-antigen reaction. This increase is generally strictly specific. The increase in viscosity reaches a maximum for a certain quantity of the added substance, which would seem to correspond to an equivalence zone. Recall injections act, as in the case of well known antigens (135 b). It is the globulins which react and Loiseleur calls them antibodies and the injected products antigens. At least in certain cases the addition of these antigens to the serum provokes a turbidity which often corresponds to the maximum of viscosity. It is necessary to add that these increases of viscosity are in general small, often of the order of 5 per cent, rarely of 10 to 15 per cent. Loiseleur thinks that in flooding the organism with a substance of low molecular weight the globulins are modified and adapted to the structure of the injected substance, which then is quickly eliminated. To prove it he injects into these animals, over a period of some hours, sufficient quantities of one of these products. He then bleeds the animal immediately and subjects this serum (which does not react with the injected substance) to a prolonged acid dialysis, in order to eliminate the injected substance. At the end of the dialysis, the serum shows a specific increase in the viscosity upon addition of the simple antigens. Loiseleur concludes from this that a transformation of the globulins has been made in the circulating blood and claims a similar mechanism to that proposed by Pauling. Doerr (18, vol. III, pp. 2 to 17) submits these experiments and, above all, their interpretation to severe criticism, as he does those of Pauling & Campbell (311) on the formation of antibodies *in vitro*, basing his argument in part on previous publications of Haurowitz (312) and of Kuzin & Nevraeva (313) who did not succeed in reproducing the results of Pauling.

We know, since Landsteiner, that molecules of low molecular weight can become antigenic when they have the property of reacting easily with proteins. Recent studies (136, 137) with such products have shown that they indeed stimulate antibody production even with resultant precipitin reactions. In a like manner studies on the action of phosgene (138) or of mustard gas (139) have proved that antibodies form following the action of newly formed antigens which result from the reaction between the gas and the proteins of the tissues. Ong (138) establishes that protection against phosgene appears in 24 hr. and lasts 3 to 4 months. Let us cite in this respect also the action of carcinogens (see below), the existence of a positive complement fixation for sulphonamides with sera of certain rabbits treated with these substances (140), the increasing insensitivity to the temperature-lowering effect of histamine after 12 days of injection into mice (141), the anaphylactic sensitizing action of penicillin and of trichophytin (142), and the numerous cases of sensitization towards substances that may even be very simple. Considering all these results, is the only admissible explanation that of the modification of normal proteins to foreign, or may these small molecules act

as antigens by themselves? Heidelberger admitted the former explanation in the course of the published discussion in Copenhagen, but added that in the case of the experiments of Loiseleur, it might be a matter of "univalent" antibodies (22, p. 328). At first sight it appears difficult to imagine a modification of proteins, particularly by certain of the substances utilized by Loiseleur, but we know that proteins react easily to a great extent. It suffices, for example, to add glutathione to ovalbumin to obtain a modified precipitin curve with antiovalbumin (310). Another troubling fact is the rapidity of appearance of the antibodies of Loiseleur, but we have already cited other cases in which the reactions of the organism have been equally rapid (119, 138). In summary, the experiments of Loiseleur merit being repeated, using other techniques, while interpretations should be reviewed in the light of well known facts. The author thinks that his hypothesis on the formation of more or less specific "carriers" would explain certain of these facts.

Nonmicrobial antigens.—The antigenicity of proteins coupled with a haptene decreases by treatment with too much haptene (143). The study of various artificial antigens led Haurowitz to the following conclusions: the specificity depends upon "the polar character of the determinant groups, their accessibility, the rigidity of the antigen molecule, and the distance between two or more polar groups of a different type" (23, p. 459). The study of the specific precipitation of artificial antigens elucidated the spatial configuration of *p*-azosuccinanilate as the *cis* form (144) and permitted a comparative study of organic compounds having closed chain or pseudo-chain structures (145). Confirming other research, Went & Kesztyus (22, p. 338) found that the injection of histamine conjugated to proteins protects the guinea pig against anaphylactic shock. The injection into rabbits of carcinogenic hydrocarbon-protein conjugates provokes the formation of antibodies which have been studied by quantitative precipitin reactions (146). The ingestion of carcinogenic substances by rats sometimes does (147), and sometimes does not provoke the increase of γ-globulins, depending on the carcinogenic activity of the product (148). The increase of γ-globulins has been interpreted to indicate an antibody response (147). A scalding of the skin of mice by sulfuric acid seems to protect the animal for some months against the appearance of tumors by injection of benzopyrene (149).

When one mixes nonantigenic lipid extracts of organs or preparations of lipids with rabbit serum and adds this mixture to pig serum, the extract does not behave as an antigen, whereas it become antigenic if one mixes the nonantigenic product directly with pig serum. Heimann (150) thinks that this invalidates the theory of the formation of a new antigen by the fixation of haptene on the proteins. It seems that one might find a correlation between these results and those obtained by the use of adjuvants, or, eventually, evidence might show the presence of natural "carriers" having different forces of combination in the sera studied. Heimann demonstrated that a certain specificity exists for lecithin, cephalin, sphingomyelin, and a glucoside of the cow's brain (150).

The use of quantitative precipitin methods enabled Cohn, Wetter & Deutsch to demonstrate minor impurities in preparations of ovalbumin and conalbumin (151) and Kaminski & Grabar to study products derived from ovalbumin by denaturation, by blocking of certain groups and by digestion. Plakalbumin, which has only six amino acids less than ovalbumin, gives a different precipitin curve (152). Isotopes P^{32} and S^{35} have been utilized in the study of immunological reactions (153). The phosphorylation of ovalbumin by $POCl_3$ gives a product which spontaneously dephosphorylates itself and which does not possess a new specificity as a result of phosphorylation (153).

Continuing the study of the formation of complexes between proteins by heat, Kleczkowski established that when the complex serum albumin-bushy stunt virus has a ratio of constituents (albumin:virus) greater than three, it is no longer precipitable by the antibushy-stunt virus sera, but still combines with antibody (154). The antigenicity of certain proteins treated with alkali (155 a), with periodate (155 b), with formalin and heat (156), or with reducing agents (157), has been studied.

Naylor, utilizing the technique of the "isochrones" of Boyd, has been able to distinguish in rabbit antihorse serum four different antigen-antibody systems (158), while Oudin finds at least eight (see below).

The use of quantitative immunochemical methods has been proposed for the determination of serum albumin (159) and of human γ-globulin. In the latter case (160, 161) fractions II (1, 2) and II-3 purified by the ethanol technique have been used. Although these contain globulins with different physical properties (solubility, mobilities, sedimentation constants) they have given identical precipitation curves, at least in the zone of antibody excess, which would seem sufficient to allow their use for clinical assay (161). However, Cohn *et al.*, after immunizing rabbits and chickens with highly purified fractions of γ_1- and γ_2-globulins, obtained sera which gave different precipitin curves, i.e., with γ_1- and γ_2-globulins (162). It seems that, with the exception of the γ_2-pseudoglobulins, which appear immunologically a little more homogeneous, all the preparations are mixtures of similar substances (see above). From this fact, in a statistical way, the curves of Kabat & Murray, obtained with different preparations, are superimposable in the zone of antibody excess. Let us mention, moreover, that comparisons of the assay for γ-globulins in sera by electrophoresis and with the help of immune sera have shown a very great difference (160). This confirms that there is no necessary correspondence between immunologic specificities and electrophoretic properties and that in each large group of globulins, characterized by a sufficiently general property (molecular weight, electrical properties, or antigenic specificity) there exists a series of similar molecules which can in certain cases be distinguished by finer techniques or be differentiated by methods based on other properties than the one that was used to define them (20).

The antigenic specificity of syphilitic globulins has been studied by Henriksen (163) utilizing the specific flocculates of a syphilitic reaction as

antigen. The serum of injected rabbits contained two kinds of antibody: one reacted with "lipid" antigen, and the other with globulins of normal human serum. Unpublished results obtained in this laboratory by Maupin showed that the syphilitic "reagin" is not antigenically homogeneous by the Oudin technique.

Russian authors found that human, guinea pig, and mouse tissue, as well as horse plasma, extracted with trichloroacetic acid by the method of Boivin contain weakly antigenic polysaccharide complexes specific for the species. The complex degraded in phenol (Morgan's method) liberates a carbohydrate which, coupled with another protein, restores antigenic specificity. The amount of this carbohydrate-protein complex in tissues is from 0.2 to 1 per cent (164).

Cytochrome-*c* of the horse is antigenic for the guinea pig (165). Ricin has been isolated in the crystalline state; the product, of mol. wt. 36,000, appeared to be homogeneous in electrophoresis and ultracentrifugation, but not by the solubility test (166 a). Preparations of purified ricin possess the properties of a protease; during fractionation, the toxicity and protease activity go hand in hand. It can hydrolyse pepsin (166 b). Rabbit antilysozyme inhibits the activity of lysozyme on *Micrococcus lysodeiktikus* (167). Papain injected in the guinea pig has not provoked the formation of antibody neutralizing its activity (168), but papain inactivated by formalin provoked the formation of neutralizing antibody to its enzymatic activity (169). The neutralizing activity of antibody precipitating D-glyceraldehyde-3-phosphate dehydrogenase, obtained in the rabbit and the chicken, can be inhibited by the diphosphopyridine-nucleotide (170).

If there still exists doubt that the antiprotein-hormones are true antibodies, because specific precipitation (with the exception of thyroglobulin) has not been observed, these hesitations should disappear, for it has been possible to demonstrate precipitation with two more hormones: on the one hand, with human chorionic gonadotrophin,[3] where the physiologically neutral specific precipitate, after treatment with alcohol to denature the antibody, liberated the hormonal activity (125); and, on the other hand, where an immune serum gave a precipitin reaction with adrenocorticotrophic hormone in solution and an agglutination reaction when the hormone was adsorbed on collodion particles (172). Antisera, preventing the hormonal response of the comb of the capon, have been prepared by injecting extracts of the comb of the cock (129).

Antigenic differences between leukocytes of lymphoid leukemia and of leuko- or lymphosarcoma have been demonstrated with the aid of specific sera (173 a), whereas cytoplasmic fractions extracted from the spleen of normal and leukemic mice did not show a strict specificity (173 b). The anti-

[3] This hormone, specifically its carbohydrate fraction, often gives cross reactions with blood group A substance, the polysaccharides of pneumococcus type XIV, and of *B. anthracis* (171).

genic properties of the constituents of nuclei (174), the role of nucleic acid in the antigenicity of the carcinogenic milk agent of the mouse (176), and the antigenic composition of the nucleo-protein constituents of the tissues of normal adult mice, and of embryonic and tumorous tissues, have been studied. Antigenic specificity of the organs and differences in antigenic composition of "embryo" and "adult" spleen and kidney have been demonstrated (177 a). Antisera against human tumors have been prepared and the authors claim that there exist specific antigens in these materials (177 b).

Attempts at cutaneous homografts in the rabbit did not succeed on a second try in the same rabbit. It seems that there was a formation of antibody, but Medawar feels that the humoral antibodies do not explain all the facts observed (178). Some new results have been obtained in the comparative study of antigens in the course of development of the chick (179). In frog eggs and in serum there exist similar but not identical antigenic substances (180).

Highly purified influenza virus coming from a culture in the egg or in the lung of mice, give reactions with sera specific for these tissues. The purified virus contains 20 to 30 per cent of host proteins (181). Quantitative assays for constituents of the egg have been performed also on antiencephalomyelitis vaccine with the help of specific sera (182).

The fractionation and study by diverse methods of extracts of ragweed pollen (183) and of cotton seeds (184) have been continued. Often several fractions are active, which increases the difficulty of these studies. The passive sensitivity (reaction of Prausnitz-Kûstner) permitted the demonstration of allergens in the honey and in the poison of bees. An infection favors the sensitization (185).

Toxins.—Recent research has shown that toxins are either enzymes themselves or act upon enzyme systems, a fact which can explain their high activities.

Formalin, acting on proteins, provokes the formation of methylene bridges between amino groups and CH groups of phenols, imidazoles, and indoles. Reactions of this sort can take place in the detoxification of toxins (186). Under the action of x-rays, toxicity and antigenicity of toxins disappear simultaneously, probably through free oxidizing radicals (187).

The extraction of washed microbial cells with citrate at a pH of 6.3 permitted Raynaud (188) to obtain appreciable quantities of toxins in a relatively pure state from *tetani, botulinum* A and B and *Clostridium sordellii*, but analogous attempts with *Corynebacterium diphtheriae, Clostridium septicum, histolyticum* and *oedematiens* failed.

In order to avoid secondary reactions during immunization of adults to diphtheria, Pappenheimer *et al.* have proposed a scheme of Schick testing and immunization using purified toxoid (189). Ross (190) proposes the use of digestion products of human serum to prepare the toxin for the Schick test. Diphtheria toxoid has been isolated pure by use of alcohol at low temperature (191). The electrophoretic diagrams of less pure preparations of toxoid

have generally shown two boundaries (192). Paper chromatography of hydrolysates of toxin and toxoid indicates that the amino grouping of tyrosine is free in the toxin and could react with formalin (193). In order to prove that the action of diphtheria toxin is related to cytochrome-*b*, research has been continued which shows that the major difference in behavior between the cytochrome system in the Bacillus and in mammalian heart muscle is the relative quantity of the different constituents (194). Studies on the fixation of diphtheria toxin in tissues permitted the demonstration that cholesterol has no effect (195 a). The diphtheria toxin does not possess its own enzymatic activity (195 b).

Tetanus toxin has been crystallized (196), but even when preserved at 0°C. it loses in ten days 75 per cent of its toxicity and its sedimentation constant increases from 4.5 to 7.5 (197). The tetanus toxin is adsorbed more easily on nerve tissue than on carbon and the adsorption on carbon does not occur in the presence of blood serum (198).

The physicochemical properties of botulinus toxins of types B (199) and A have been studied (200). The crystallized type A toxin has a molecular weight of 900,000, a frictional constant of 1.76 and is serologically homogeneous (200). The type D toxin extracted from microbial bodies is different from toxins A and B. The microbe produces, in addition, a nonantigenic substance which produces convulsions in animals (201). The botulinus toxins seem to intervene in the acetylcholine system (202, 203).

Numerous publications relate to the toxins of gas gangrene: purification, thermostability, distribution in diverse species of microbes, flocculation reaction, immunological comparison and enzymatic activity (lecithinase, collagenase, hyaluronidase, hemolytic effect of lysolecithin, etc. (204).

Staphylococcal toxin has been purified 200-fold by precipitation with methyl alcohol under precise conditions of pH, temperature, and ionic strength (205). It seems that there is an inverse relationship in the formation of β-toxin and the fibrinolytic power of different strains of staphylococci (206, 207). There seem to be at least two antigenically different lysins (208). The fibrinolytic activity of the staphylococcus is analogous to that of streptococcus (kinase), and the staphylocoagulase is an independent constituent (209).

The properties of streptolysins and their comparison with toxins of other microbes were reviewed in 1948 (210). The presence of a desoxyribonuclease, distinct from the streptokinase, and the methods of measuring their activities, have been described (211).

Hyaluronic acid and hyaluronidase, in the case of streptococci (212) and pneumococcal pneumonia (213), have been studied with relation to the virulence and protective effect of antihyaluronidase. Let us mention in this respect that one can render a nonvirulent strain of rabies virulent by means of hyaluronidase which exists in the saliva of dogs (214).

Microbial antigens and haptenes.—A general review on the cross immunities and the cross reactions between bacteria, virus, protozoa, moulds, and nonmicrobial antigens has been published in Brazil (215).

Among the methods utilized for the extraction of microbial antigens, the utilization of ultrasonic techniques is becoming more widespread. It presents numerous advantages, but it must not be forgotten that in addition to mechanical action, powerful ultrasonic vibrations have chemical effects as well which can modify the antigens. A detailed study of this problem made in this laboratory showed that ultrasonics can provoke the formation of free (OH) radicals from water, resulting in the modification of aromatic nuclei (216). The mechanical effect acts on particles of the order of 15 mμ or larger, as studied on phages of different dimensions [Rouyer, Prudhomme & Grabar (22, p. 330)]. One can sometimes protect the substance against the oxidizing effect of ultrasonics by an atmosphere of hydrogen, or, even better, by adding traces of ether which seems to inhibit the electrical discharges between the walls of the bubbles of gaseous cavitation (217).

The properties of antigens T (218) and M of the streptococcus have been studied: the latter plays an important rôle in virulence against the mouse (212) and is released into culture filtrates only by the less virulent strains (219). Ultrasonic extracts of streptococci, fractionated by centrifugation, have been utilized for the study of sera of rheumatic and scarlet fever patients (220). Group D streptococcus has a capsular antigen which resists trypsin and heat (221).

The antigen common to all gonococci is thermostable, whereas the group-specific antigens differ in their resistance to heat and to preservation (222).

Measurements by ultracentrifugation, diffusion (223), and electrophoresis (224) of purified pneumococcal polysaccharide have shown that they are heterogeneous and polydisperse. By fractionation, one can obtain electrophoretically more homogeneous fractions (224).

In addition to the fact that these capsular pneumococcal polysaccharides are antigenic for man and provoke the appearance of quantities of antibodies comparable to those one observes in rabbits which have received a single injection of pneumococcus (225), another fact has just been cited by Heidelberger & Di Lapi (23, p. 444) which provoked a debate at the Cambridge Congress on a fundamental problem of immunology,—that of the persistence of the immunity. It has been established that in man immunized with polysaccharides the titer of antibodies is maintained for three years at 50 to 60 per cent of the initial titre and a reinjection does not cause it to increase. Is this a question of the persistence of the antigen in the organism with resultant saturation of the capacity to produce antibody, or does it involve a permanent specific formative mechanism of antibody production? Progress in this field is awaited with great interest.

The carbohydrate P-10 of *Serratia marcescens*, which is in reality a lipopolysaccharide antigenic for mice, loses its toxicity by heat, but provokes the formation of precipitating and protecting antibody (226). The dextran synthesized by *Betacoccus arabinosaceous* and the levan of *Pseudomonas mors-prunorum* have been characterized (227). A polyuronic acid, prepared from cellulose, coupled to proteins provoked the formation of specific antibodies which did not react with the pneumococcus (228).

Recent reviews on the carbohydrates of the *M. tuberculosis* show the complexity of these materials (229). The principal sugars found are: D-arabinose, D-mannose, D-galactose, L-rhamnose, and probably D-glucosamine [Stacey (23, p. 472)]. The oily extract of tubercle bacilli contains a protein which sensitizes to tuberculin and a lipocarbohydrate antigen which also participates in acid-resistance (230). The acid-resistance of the organisms would seem to be due to the carboxyl grouping of the mycolic acids (231).

In continuing the research on tuberculin, Seibert described a method of separation of three protein fractions and two polysaccharides which she studied by electrophoresis (232). Two distinct constituents, and sometimes a third, have been revealed by immunological techniques (233). The rôle of wax in anaphylactic hypersensitivity has been studied (234). Ultrasonic extracts of tubercle bacilli contain a glucoprotein antigen (235). Microbes killed by ultrasonic vibrations might be used in the preparation of a vaccine (236).

For a long time workers have utilized mucin to increase the virulence of certain microbes for laboratory animals. Although this method can be criticized from the theoretical point of view, it often renders appreciable service. A recent review summarizes the question (237). A very active fraction has been extracted from gastric mucin of the pig (238).

The toxicity of the endotoxins of the group of typhi-dysenteric-choleric bacteria, etc., which are also excellent antigens, has not permitted their practical utilization in immunization and the attempts at detoxication have been without success. However, Treffers, in acetylating with acetic anhydride the somatic antigens of *Shigella dysenteriae* and of *Salmonella typhi*, has reduced to a great extent their toxicity, without making them completely lose their antigenic properties; the animals injected were slightly protected but had not formed agglutinins (239). Quinine appears to have an antitoxic effect (240). It seems that there exists beside the endotoxin a labile antigen, a neurotoxin, in the *S. typhi* (241), but irrefutable proof has not yet been found. A total of 0.1 μg of antigen O injected in ten doses in rabbits suffices to provoke the appearance of agglutinins. The O polysaccharide can inhibit the precipitin reaction of the complete antigen by immune serum of the rabbit, if it be used in a sufficiently high concentration (2 per cent). These attempts seem to confirm that the specificity of the complete antigen is due to the O polysaccharide. The latter polysaccharide coupled to a protein has provoked, in the rabbit, the formation of antibody which precipitates the complete antigen, but not the polysaccharide alone (242). The injection of cow milk in rabbits provokes the formation of antibody to O and Vi antigens which might be due to the existence of cross-reacting substances (243). On the effects of the typhoid and dysenteric endotoxins *in vivo* see (244). Four labile antigens having different toxic properties seem to exist in *Shigella*, the hypothermic effect being due to different fractions (245). The polysaccharide of this microbe contains galactose, rhamnose, and glucosamine (246). The saliva of dysentery patients contains a specific antigen demonstrable by specific

precipitation (247). The meningococcus endotoxin inhibits the phosphorylation of glucose *in vitro* (248). The exhaustion of the serum of typhus patients by *Proteus* OX19, does not eliminate the specific agglutinins of the rickettsia (249). Utilizing the method of Gard for the separation of the flagella, Weibull (250) studied the protein flagella antigens of the *Proteus*, their chemical composition, molecular dimensions, and, with Astbury, diffraction of x-rays (23, p. 149) etc. The pathogenic power of different strains of *E. coli* seems to depend upon the presence of the capsular antigen K (251). The extraction of protective antigens of *Brucella* has been described (252), as well as the preparation of vaccines with the help of ultrasonics (253).

The hypothermic substance separated from the cholera endotoxin is nonantigenic (254). A polysaccharide isolated by the aid of phenol extraction, protects the mouse against the injection of 200 MLD of vibrions (255). A "filtrate factor," similar in its action to the cholera toxin, has been isolated from choleric filtrates (256).

Extracts of *Haemophilus pertussis*, prepared with the help of ultrasonics, freezing and thawing, or urea, have served for the isolation of antigens 257, 258). In addition to the agglutinogen and toxic fractions already known, they contain a hemagglutinin (257, 259 a, 259 c). Experiments done in this laboratory showed that hemagglutinin is very rapidly destroyed by ultrasonics, even in the presence of hydrogen (see above); the toxin can be protected by hydrogen, whereas the agglutinogen is stable even in air (257). Hemagglutinin is said to be a good protective antigen for mice (259 b).

Parallel research undertaken by three independent groups of workers in France, U.S.A., and Great Britain during and after the war on *Bacillus anthracis* have been summarized recently (1). Since then, Gladstone (260) described the production of the protective antigen in cellophane sacs and Staub (261) continued the study of the protective antibody of horse serum with the aid of the antigen of Gladstone. She confirms that this antibody is a pseudoglobulin and thinks that the protective antigen is not a glucoprotein. The American group studied mainly the factor provoking inflammation and the relation of this substance to the capsular polypeptide and to the agressine of Bail as well as the substances which inhibit the growth of *B. anthracis* [histone and cationic detergents (262)].

Miscellaneous works have been reported upon as follows: on the antigenic constituents of lactobacillus of the human mouth (263); on the antigenic structure of the spores of some bacteria (264); on the Gram complex of microorganisms and of animals (265); on the antigenic components of the psittacosis virus (266); on the quantitative reactions of immune serum with different preparations of plant virus, as a function of virulence and of homogeneity of the strains (267); the toxicity of the mushroom, *Fusarium sporotrichoides* (268), the immunological relations of the spirochetes (269); and the toxic components and the antigens in extracts of *Ascaris* (270).

Venoms.—A monograph on snake venoms and antivenoms has been published by Boquet (271) who summarized his research in this field. Quantita-

tive agreement has been found between flocculation and neutralization by the antivenoms (272).

Toxic constituents and proteases of the venoms of *Crotalus* and *Bothrops* have been isolated and studied with the aid of electrophoresis (273). There has been demonstrated in 14 out of 16 snake venoms an ophio-L-amino-oxidase which activates the tissue proteases of the bitten animal (274). Inactivation by thio-derivatives and cyanide makes one think of the necessity of disulfide groups for the toxicity (275). Several different activities of the venoms have been described: hyperglycemic action of the venom of *Naja* and of *Bitis* (276); hemagglutination (277) and destruction of complement (278) by the venom of *Bothrops;* and nondestruction of complement by the venoms of *Microrus* and *Crotalus*. Studies on the poisons of scorpions of different countries have also been published (279).

Antigen-Antibody Reaction

Theoretical aspects.—Isohemagglutinins have been determined in relative values according to the maximum number of agglutinated red blood corpuscles under defined conditions. The reaction is reversible and exothermic The curves established at two different temperatures permit the calculation of an enthalpy of $-19{,}000$ calories, which would correspond, for example, to 3 to 4 hydrogen bonds per molecule of agglutinin (280). In utilizing coupled antigens with the bis-azo-phenyl carboxylic grouping, Haurowitz & Etili observed that in the presence of antigen excess the specific precipitate is dissociated and there are 6 to 8 molecules of antibody liberated. From the area of active sites, they calculate that a bond between the antigen and the antibody corresponds to approximately 0.47 kcal/Å^2. The bindings which are easily dissociated correspond to less energy of the order of 0.25 to 0.10 kcal/Å^2 (281).

The calculations of Teorell, based on the hypothesis of monovalent antibodies, permitted him to develop theoretical precipitin curves which greatly resembled curves obtained experimentally (283); but this agreement does not prove the monovalence of antibodies. Using Teorell's model and assuming also that antibodies are monovalent and antigens polyvalent, and that both are spherical molecules, mathematical calculations have been made to characterize the equilibrium reactions between the two substances. The free energy change per individual weak bond appears to be of about $-1{,}400$ cal. (282). But all these calculations, often being based on assumptions which may not be very exact, although interesting, may be useless. Utilizing the lipovitellin precipitin system, containing isotope P^{32} and antibody with radioactive iodine, as well as other labelled antigens and heavy water, an attempt has been made to determine the valence of antibodies; the results would seem to be in favor of monovalence but the technical difficulties allow a certain doubt to remain (284). The electrophoretic study of solutions of specific precipitates in antigen excess (serum albumin and ovalbumin) pleads,

on the contrary, in favor of a bivalence of antibody [Marrack (23, p. 449)], as does the study of the combination of purified antibody with homologous haptene dyes, as a function of their concentration (285).

An interesting discussion between defenders of the theory of the monovalence and the proponents of the theory of the multivalence of antibodies took place in Cambridge after the communications of Marrack (23, p. 449) and of Wormall (284). It seems that new arguments should be brought to bear on the question. It would be desirable that the new knowledge acquired on the structure of proteins be taken into account instead of designing circles or imaginary figures. If all the globular proteins have a rigid structure analogous to that which was recently described for hemoglobin (four planes of peptide chains superimposed), one can envisage either that the two planes turned toward the exterior (the superior and the inferior) are identical and in this case the molecules would be bivalent in some way, or that the two planes are not necessarily identical and thus one can envisage variants between monovalence and bivalence. This representation of the protein molecule as a sufficiently rigid structure appears to be in contradiction to the conception of Pauling who represents it as a sort of bundle formed by the folding of a single peptide chain. If the results of Porter (23, p. 142) are confirmed, they might be interpreted as an argument in favor of a single peptide chain in the molecule of the antibody, but the absence of several terminal groupings may just as well signify that the peptide chains are closed on themselves. Once more, progress in the knowledge of the structure of proteins would facilitate the comprehension of immunological reactions.

Study of the reaction of antibody to *p*-azosuccinanilate with its antigen and 50 inhibitor haptenes has led to the conclusion that the forces of attraction between antibody and haptene result from the charge of the carboxyl groupings and from the Van der Waal attraction forces of the benzene ring (286). In response to previous criticisms, measurements of degree of association in solution of various simple haptenes have been made. They vary from 1.3 to 400 (287). There must be taken into account the possibility of an association as an explanation of the precipitin reaction or turbidity observed even with small molecules, a phenomenon discussed previously.

After an analysis of the mechanism of immune hemolysis, Morris was led to envisage the heterogeneity of the erythrocytes, and made a hypothesis on the number of molecules of antibody fixed by an erythrocyte (288).

Rothen (289) continued his research on "long range forces" through multimolecular layers by allowing enzymes to act on films of antigens. The results of his research have been criticized. Karush & Siegel think that at the time of drying of the protein films, aggregates formed which emerged later through outer layers (290). The different behavior of ovalbumin and of serum albumin could be explained by the greater facility of denaturation of the former, the layers presenting different aggregates. Shulman, at the Cambridge Congress, also affirmed that the layers are never perfectly united and always have holes. The observations of Rothen might be explained by

the passage of substances through holes of the successive films and not by a "long range force."

Methods and applications.—A review of theoretical and practical aspects of precipitin reactions was published by Neuzil (291).

The utilization of quantitative methods of specific precipitation has found importance in more and more fields of research, many cited previously. They permit not only the determination of the amount of antibody in serum, but also the comparison of the amount of antigens in various unknown mixtures; thus one can follow the purification, the degradation, or the modification of an antigenic substance, etc. Furthermore, specific precipitation may provide another criterion of homogeneity of a preparation. The form of the curve in the zone of antigen excess has been considered by Pappenheimer and by Deutsch *et al.* as being able to furnish information of this nature (88, 151). Studying the quantitative precipitation of diphtheria toxin by rabbit and human antitoxin, these authors established that if the quantitative precipitin curve extends into the inhibition zone, there is an indication of secondary antigens in the system; if, on the contrary, the protein antigen is pure, the curve falls rapidly to zero. Analogous reasons applied to the study of conalbumin and of γ-globulins have often led to useful results (151, 162). We must not forget, however, that there are other factors which influence the path of the curve, such as the solubility and physical form of the antigen. In the antigen-excess zone, particularly for nonproteins and certain complex substances, i.e., somatic antigen, the solubility of the complex depends above all on the solubility of the antigen. It has been demonstrated previously that the constituents formed, even by ovalbumin, in the zone of inhibition have different solubilities [studied by solubility in low concentration of alcohol (292)]. If the antigen is only slightly soluble itself, the curve tends to be lengthened. The same is true if, by a reaction, one renders the antigen less soluble. Thus, ovalbumin coupled with a haptene by diazotization gives, in the zone of antigen excess, less and less sharp curves as a function of the quantity of fixed dye which renders the ovalbumin less soluble (unpublished experiments). It is not impossible that certain differences observed in the form of the precipitin curves of γ_1- and γ_2-globulins (162) are due only to the fact that the preparations of γ_1-globulins were above all euglobulins, therefore less soluble; but, as we have said, there are also differences of another order between the two fractions.

There are cases in which the quantitative method appears to be limited. Thus in the study of the precipitin reaction of preparations of lecithinase of *Clostridium welchii* at two different stages of purification Van Heyningen & Bidwell found aberrations which they interpreted as nonspecific adsorption by the specific precipitate (293). The co-precipitation of the α-globulins of the immune sera of chickens has already been mentioned above. It does not seem to be a question here of a nonprecipitating antibody (43). In certain cases one can claim that there can be adsorption of substances as, for example, dyes or other substances by the specific precipitate (294), or mutual precipi-

tations, as azoproteins with noncoupled proteins in acid solutions (295) or guanidine denatured γ-globulins with native protein (296) or of the botulinus toxin, type A, with the proteins of normal serum (297) or of pepsin with split diphtheria antitoxin, which resembles a specific precipitate (298). In other cases, one can explain the reactions or adsorptions called "nonspecific" by the existence of cross reactions [e.g., the case of the antilipovitellin antibody which takes phospholipids into the precipitate (284)], and, finally, by the presence in the serum of nonprecipitable antibody or of "carriers" which can unite to the components of antigen without giving a precipitate, but which are trapped in the precipitate formed by precipitating antibodies. It is necessary therefore when one wants to utilize the quantitative method, to take some precautions to avoid errors.

The utilization of agar gels for the study of reactions of precipitation has been envisaged by two authors. The principle involved is simple: one incorporates one of the reagents (immune serum or antigen) into a gel and one lets the other reagent diffuse into it. The meeting of the two substances in suitable proportions provokes the formation of a precipitate which forms an opaque layer in the gel. Oudin (299) utilizes tubes in his method. The diffusion occurs principally in a downward direction and one observes the formation of a horizontal layer of precipitate when there is only one antigen and one antibody. If the system consists of several precipitant systems, there are as many layers as there are systems. One can, thus, in a mixture of unknown antigens, determine the minimum number of different antigens. Cross reactions have also been studied, as well as the relative diffusion speeds of the different constituents as a function of their concentration, of the temperature, etc. Ouchterlony utilizes Petri dishes containing a layer of the gel with the horse immune serum. The antigen is placed in a well formed in the gel. The diffusion takes place horizontally and the precipitate is formed as crowns or rings around the well. If one places identical antigens in two neighboring wells the precipitates form a common crown, and if the antigens are different the crowns independently intercross (300).

It seems that the technique of Oudin lends itself better to the enumeration of antigens in a mixture (at least eight different antigens have been demonstrated as present in horse serum), and it is possible that his system can be transformed into a quantitative method, whereas the technique of Ouchterlony more easily permits comparison, from the point of view of the identity of the antigens (for example, comparison of the capacity to produce diphtheria toxin from numerous microbial strains).

Two techniques have been proposed to simplify or perfect the methods already known to determine quantity of specific precipitate: the utilization of the falling drop technique has given good results for the assay of pneumococcal polysaccharide (301); the utilization of ultraviolet spectroscopy also permits assay of the quantity of proteins (antibody) in a specific precipitate (302). Boroff & Tripp (303) utilize the specific aggregation of streptococcal proteins adsorbed on oil droplets in the study of these proteins.

In a series of publications Hole & Coombs (304) have described in detail the phenomenon of conglutination, methods employed, results in certain maladies, and the choice of the type of animal furnishing the complement. Certain reactions fix complement only in one species of animal. In the titration of blocking Rh antibodies, serum albumin can be replaced by diverse gums (305). When one wants to titrate typhoid agglutinins of plasma, the choice of the anticoagulant is important, for certain anticoagulants appreciably diminish the titer (306). There are differences in the optimum temperature for demonstrating the antityphoid agglutinins: 4° for the "normal" agglutinins and 37° for the agglutinins of the immunized animals (307).

New details have been presented by Neurath *et al.* (109) on the use of a thermostable inhibitor of "false positive" syphilitic reactions of the serum which permits one to distinguish the true positive reactions.

Toxoplasma placed in the presence of an immune serum are abnormally colored with methylene blue; this difference in coloration may be used to explain the action of immune sera on different parasites (308).

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BIOCHEMISTRY OF ANTIBIOTICS[1]

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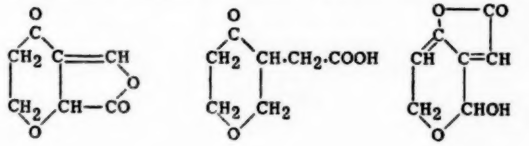
The volume of the literature in the antibiotic field has made it necessary to limit the scope of this review. Those aspects which are covered in the *Annual Review of Microbiology* will be touched on lightly, if at all. A brief section dealing with antibacterial substances from higher plants has been included since several interesting papers have appeared in this area. Of outstanding interest during the past year have been the rapid development in the clinical use of aureomycin and chloramphenicol, and the recognition of their action on certain larger viruses; the many improvements and modifications of the tools for detecting, characterizing, and purifying antibiotic substances (paper chromatograms, counter-current distribution, ion-exchange, partition, and adsorption columns); the elucidation of the structure of chloramphenicol and its synthesis (the first antibiotic to be synthesized on a commercial scale); and the constant addition of new antibiotics to the already long list.

For the most part we have followed the classification scheme based on increasing complexity of elementary composition which has been used in previous reviews.

Compounds Containing Carbon, Hydrogen, Oxygen

Patulin (Clavacin), $C_7H_6O_4$ (1).—Conclusive evidence has appeared that the originally assigned structure of patulin, I, is incorrect (2 to 5).



I II III

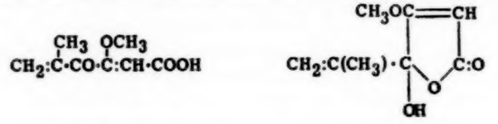
Catalytic reduction of patulin in aqueous solution yields desoxypatulinic acid (3) which has been shown by synthesis to have the structure II (2, 3). This eliminates I as a possible structure for patulin. Two alternative formulas have been proposed (2, 3). Of these III seems preferable since it accounts for the hydroxyl group shown to be present by infrared absorption (5) and the fact that patulin phenylhydrazone still retains the doubly-unsaturated lactone ultraviolet spectrum (5). Finally, Woodward & Singh (2) synthe-

[1] This review covers the period from approximately December, 1948 to December, 1949.

sized III minus the hydroxyl group (desoxypatulin) and reported that it had an absorption spectrum almost identical with that of patulin.

The high toxicity of patulin is illustrated in recent reports. At a dilution of 10^{-6} patulin inhibits mitosis of heart fibroblasts (6). A 0.1 mg. daily dose (intraperitoneally) reduced the lymphocyte count of mice from 8,000 to 15,000 to 1,000 to 3,000 (7). (The granulocyte count was not affected.) Jirovec states that patulin is one of the most toxic substances known for protozoa (8).

Penicillic acid, $C_8H_{10}O_4$ (1).—Munday (9) has reported infrared absorption spectra which were interpreted as evidence for the existence of two tautomeric forms of penicillic acid, IV and V, in the solid state. More recent work (10) has indicated that Munday's keto acid, IV, was actually the monohydrate of the lactol V. The infrared spectra of penicillic acid in chloro-



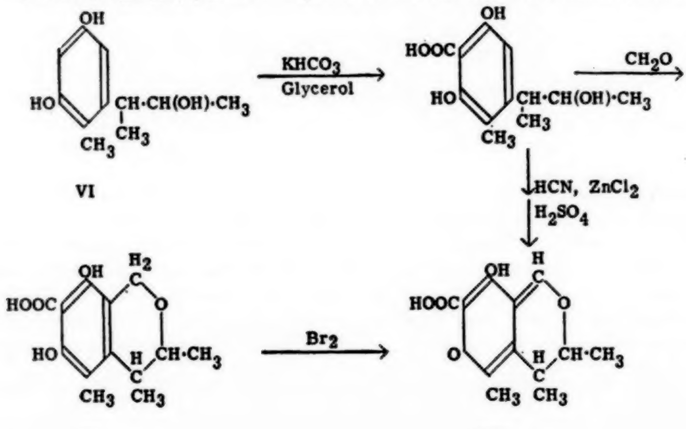
IV V

form or deuterium oxide solutions indicated a predominance of V, while the potassium salt in deuterium oxide was found to be that of the keto acid IV. Thus, the tautomeric formula which was proposed in 1936 by Birkinshaw, Oxford & Raistrick (11) appears to be correct.

A synthetic analogue in which the isopropenyl group was replaced by a phenyl group has been reported by Nineham & Raphael (12). Its antibacterial activity was about one-fourth that of penicillic acid.

Gladiolic acid, $C_{11}H_{10}O_5$ (1).—Brian *et al.* have made a detailed study of the production and biological properties of gladiolic acid (13).

Citrinin, $C_{13}H_{14}O_5$ (1).—Final proof of the structure of citrinin VIII was



VI VII VIII

afforded by its synthesis from the phenol VI (14, 15) [a degradation product of citrinin whose structure was established by synthesis (16)].

The oxidation of dihydrocitrinin VII to citrinin was discovered by Schwenk *et al.* (17) who also reported an improved method for obtaining VI by treating citrinin with ammonium hydroxide.

Trichothecin, $C_{15}H_{20}O_4$, *Rosein I, II, III* (*Rosonolactone, Rosenonolactone*), (*Trichothecium roseum Link*), $C_{19}H_{26}O_3$, $C_{19}H_{28}O_3$, $C_{20}H_{28}O_4$.—The fungus *T. roseum* Link, (causes "pink rot" in apples) grown on a modified Czapek-Dox medium containing corn steep produces several related substances. Trichothecin occurs mainly in the culture fluid (18), rosein I and II in the mycelium with small amounts in the medium, rosein II only in the culture medium (19). Other workers have obtained rosenonolactone and rosonolactone (20) from the same fungus. They are believed to be identical with rosein I and rosein II respectively.

Trichothecin has a wide range of antifungal activity (21) and is responsible for the major portion of the activity of *T. roseum*. Rosein II has some antibacterial activity, roseins I and III are inactive (19). Trichothecin is the main constituent of the bitter principle which accumulates in fruits infected with *T. roseum*.

Usnic acid, $C_{18}H_{16}O_7$ (1).—Usnic acid is produced by several species of lichens. Castle & Kubsch (22) have shown that in one case at least (*Cladonia cristatella* Tuck) the fungal component of the lichen is responsible for the production of usnic acid.

Herquein (*P. herquei*), $C_{19}H_{20}O_8$.—Herquein is a yellowish-brown crystalline solid (m.p. 129°C.) soluble in organic solvents (23). It inhibits several bacteria including *Vibrio cholera*.

Compounds Containing Carbon, Hydrogen, Oxygen, Nitrogen

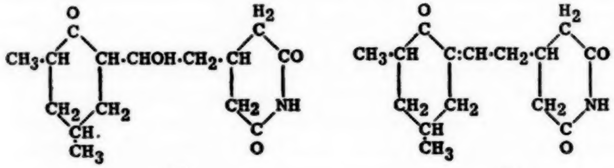
Candidulin (*Aspergillus candidus*), $C_{11}H_{15}O_3N$.—Candidulin was obtained from culture fluid of *A. candidus* by extraction with chloroform at pH 2, 7, or 10. It was purified by crystallization from hexane [m.p. 88 to 89°C., $[\alpha]_D = +15°$ (chloroform)]. Candidulin contains no titratable acidic or basic groups and gives no absorption in the ultraviolet (24).

Candidulin inhibits a variety of bacteria, including the *Mycobacteria*. However, it afforded no protection to mice infected with *M. tuberculosis*. This result may be due to the fact that its activity is markedly decreased by whole blood.

Cycloheximide (*Acti-dione*[2]), $C_{15}H_{23}O_4N$.—Further information concerning the structure and chemistry of cycloheximide IX has been published by Kornfeld, Jones & Parke (25). An anhydro derivative X was obtained by the action of phosphorus pentoxide in benzene, and catalytic hydrogenation led to the formation of a dihydro derivative in which the carbonyl group of IX was reduced to a secondary alcohol. Oxidation of IX with chromium trioxide led to the formation of a 1,3-diketone. The C_8 ketone which had been ob-

[2] Cycloheximide is the trivial name for Fig. IX. Acti-dione is now the registered trademark of The Upjohn Company for this compound.

tained by alkaline degradation was identified as 2,4-dimethylcyclohexanone by comparison of its infrared spectrum with those of the synthetic compound and some closely related isomers. The C_7 fragment which was obtained by alkaline hydrolysis, presumably propionaldehyde-2,2-diacetic acid, was not isolated in pure form but was oxidized to methanetriacetic acid. Among the

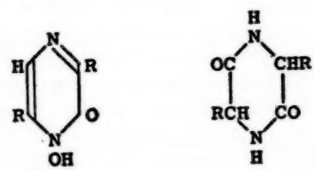


IX X

evidence for the glutarimide ring was the fact that the electrometric titration revealed the presence of a weakly acidic group whose pK_a' value, 11.2, was found to be the same as that of glutarimide. Physical evidence that the remainder of the molecule was attached to the β-position of the glutarimide ring was obtained by comparing the infrared spectrum of cycloheximide with those of synthetic α-ethylglutarimide and β-ethylglutarimide. This appears to be the first record of a natural product containing the glutarimide ring.

Clinical investigations which are now in progress indicate that cycloheximide may prove to be of value in the treatment of a systemic fungus infection, cryptococcosis (26), a rare but usually fatal disease. Cycloheximide has been found to be active against *Endamoeba histolytica* in concentrations as low as 0.1 mg. per l. (27). The discovery by Felber & Hamner (28) that very dilute solutions of cycloheximide (1 to 5 mg. per l.) arrested the growth of mildew (*Erysiphe polygoni* D.C.) on bean plants has been followed up by Vaughn, Lockwood, Randwa & Hamner (29) who have reported that the high fungicidal activity extends to a number of other plant pathogens. As was found to be the case in its toxicity to mammals, there is apparently a wide variation in its toxicity to various species of plants.

Aspergillic acid, $C_{12}H_{20}O_2N_2$ (1).—In a study of the structure of aspergillic acid, XI, Dunn *et al.* (30, 31, 32) have shown that the diketopiperazine, XII, obtained as a degradation product, is derived from one molecule of DL-leucine and one of isoleucine (or *allo*-isoleucine).



XI ($R=C_4H_9$) XII ($R=C_4H_9$)

For comparison with XII the anhydrides of DL-leucyl-DL-isoleucine, DL-isoleucyl-DL-isoleucine, and DL-norleucyl-DL-isoleucine were prepared (31). Each of these compounds melts in the range 255 to 282° and gives no depression of melting point with XII. Hydrolysis of XII gave DL-leucine and a second amino acid giving methylethylacetaldehyde with ninhydrin (32).

Absorption spectra of related pyridine derivatives show that aspergillic acid has the pyrazine hydroxamic acid structure, XI, rather than the isomeric 2-hydroxy-N-oxide structure (33, 34). The hydroxamic acid formulation is also supported by the stability of aspergillic acid (35).

Methods for the synthesis of pyrazine hydroxamic acids (36) and their pyridine (33, 35, 37), pyrimidine (35), and quinoline (33, 35, 37) analogues have been developed. Several of these compounds have antibacterial activity comparable to that of aspergillic acid.

Streptomycin, $C_{21}H_{39}O_{12}N_7$ (1, 38).—The chemistry of streptomycin has been covered thoroughly in previous reviews. Only a few additional papers have appeared in the past year. Kuehl *et al.* (39) have elaborated an earlier note clearing up the last points regarding the configuration of streptose. Carter *et al.* (40) reported details of the degradation of N,N-dibenzoyl-streptamine to α,γ-diamino-β-hydroxyglutaric acid and α,γ-diaminoglutaric acid. The latter compound was synthesized as a result of the development of a new general method for the preparation of α,γ-diamino acids (41). Stavely & Fried (42) reported in detail on the stepwise degradation of mannosidostreptomycin (streptomycin B), establishing its structure as an α-D-mannoside of streptomycin with the mannose attached to carbon 4 of the N-methylglucosamine unit. The crystalline sulfate (43, 44), hydrochloride (43), and β-naphthalene sulfonate (45) of dihydrostreptomycin have been prepared. Three isomers of streptamine have been synthesized by ammonation of α- and β-dibromoinositol (46).

The reductive amination of the aldehyde group of streptomycin is readily effected. Streptomycylamine (47) and a series of N-alkylstreptomycylamines (48) have been prepared in this way.

$$\text{Streptomycin (CHO)} + RNH_2 \rightarrow \text{Streptomycylamine } (CH_2NHR)$$

The Craig distribution technique has been applied to the separation and characterization of streptomycin, mannosidostreptomycin, and other water-soluble basic antibiotics (49, 50, 51). The various systems used contained a bicarbonate (49), borate (51), or borate-phosphate buffer (50), with pentasol (mixed amyl alcohols, Sharples) as the non-aqueous phase. In order to increase solubility in the organic phase a "carrier" acid (lauric or stearic) was employed.

An analytical method for dihydrostreptomycin has been developed based on the fact that it yields 1 mole of formaldehyde on periodate oxidation (from the primary alcohol formed on reduction of streptomycin). The latter compound gives no formaldehyde with periodate (52). Perlman (53) has developed a chemical method for mannosidostreptomycin based on the determination of mannose by the carbazole reagent. A "capillary-tube" modifica-

tion of the standard agar plate bioassay procedure has been described (54, 55).

Rake *et al.* (56) have continued their studies of the *in vitro* activity of streptomycin, mannosidostreptomycin, and their dihydro derivatives. Dihydrostreptomycin has about the same antibiotic activity as streptomycin and is ineffective against streptomycin-resistant organisms (57). However, in view of its reported lower toxicity, the dihydro form has assumed considerable clinical importance, particularly in the treatment of tuberculosis (58, 59).

The production and biological properties of streptomycin-resistant and streptomycin-dependent mutants of various bacteria have been reported (60, 61, 62). In contrast to the progressive increase in resistance to penicillin, bacteria may become completely resistant to streptomycin in one exposure (63). Development of resistance to streptomycin is suppressed both *in vitro* (64) and *in vivo* (65) by *p*-aminosalicyclic acid. Fatty acid salts of streptomycin produced a markedly lower resistance in *Salmonella typhosa* than did inorganic salts (66).

Winsten reported an interesting study of the alkylstreptomycylamines (67). Dihydrostreptomycin and the shorter chain alkyl derivatives were utilized by streptomycin-dependent forms while the longer chain derivatives inhibited both the dependent and the resistant forms.

Streptothricin VI.—A substance reported as streptothricin VI on the basis of slight differences from streptothricin in antibiotic spectrum (68) has since been shown by counter-current distribution studies to be streptothricin (51).

Neomycin (*Streptomyces 3535*).—In the course of a large screening program for new antibiotic-producing actinomycetes Waksman *et al.* isolated a *Streptomycete* (No. 3535) (closely related to *S. fradiae*) which produced a new antibiotic, neomycin (69, 70, 71). The crude material was active both *in vitro* and *in vivo* against a variety of gram positive and gram negative organisms, including *Mycobacterium tuberculosis* and other mycobacteria. It had a low toxicity and was equally effective against streptomycin-resistant and sensitive strains. Neomycin-sensitive bacteria showed little or no tendency to develop resistance to it.

The crude product was later shown to be a mixture of at least three antibacterial substances (72) by the Craig counter-current distribution technique using the borate buffer-pentasol system previously described (51, 73). The term neomycin complex is suggested for the crude product. Previous to this publication Peck *et al.* (74) had isolated a homogeneous amorphous hydrochloride—neomycin A hydrochloride—from the *Streptomyces* (No. 3535) culture fluid. Whether this substance is the main component of the "neomycin complex" is not apparent since no yields were given. Neomycin A is a water-soluble base giving a positive ninhydrin test and negative tests for glucosamine and guanidine. *Streptomyces* (No. 3535) produces an antifungal agent (Factor X) (72) in addition to the antibacterial substances. Clinical testing of neomycin is now in progress.

Borrelidin (*Streptomyces rochei*), $C_{28}H_{43}O_6N$.—From a new *Streptomyces* species Berger *et al.* (75) isolated a crystalline acid with a high *in vitro* activity against *Sarcina lutea* and certain micrococci but with almost no other antibacterial activity. The name borrelidin was applied to the substance since it had a high *in vivo* activity against several strains of *Borrelia*, the relapsing fever spirochete (76).

Borrelidin is extracted from the culture fluid by butyl acetate, purified and eventually crystallized from benzene [m.p. 145 to 146°C., $[\alpha]_D = -28°$ (ethanol)]. An absorption peak at 256 mμ indicates conjugated unsaturation in the molecule.

Crude penicillin has an "enhancement factor" which increases its activity against the syphilis spirochete (77). Borrelidin possesses penicillin-enhancement activity, and from inactivated impure penicillin a substance having borrelidin-like antibiotic activity was isolated (75). However, this material was not identical with borrelidin. Substances with similar activity were isolated also from corn steep and from clarase.

Enniatin (*Lateritiin*) *and related products from Fusaria*, $C_{22}H_{38}O_6N_2$ (1).—Cook *et al.* (78) have described the isolation of a series of related products (lateritiin-I and -II, avenacein, sambucinin, and fructigenin) from various strains of fusaria, and are not willing to accept the conclusion of Plattner & Nager (79) that lateritiin-I is a mixture of enniatins A, B, C.

Antimycin A (*Streptomyces sp.*), $C_{28}H_{40}O_9N_2$.—The isolation of antimycin A from an unidentified species of *Streptomyces* has been reported by Dunshee *et al.* (80). The culture which produced this antibiotic was found as a contaminant on a plate culture of *Venturia inaequalis*, the fungus which causes apple scab (81). Antimycin A is a very potent fungicide and was reported to inhibit *Nigrospora sphaerica* at concentrations as low as 0.00125 mg. per l. The crude preparations were found to be active against some other species of fungi which are pathogenic to plants (81).

The crystalline product is dextrorotatory and colorless. It is insoluble in water and only very slightly soluble in petroleum ether, benzene, and carbon tetrachloride. It is quite soluble in alcohol, ether, acetone, and chloroform. Qualitative tests demonstrate the presence of a phenolic hydroxyl and the absence of amino or carbonyl groups.

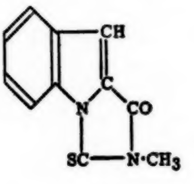
Musarin (*Streptomyces sp.*), $C_{35}H_{60}O_{14}N_2$.—In searching for antibiotics which might prove to be useful in combating fungus diseases of the banana plant (*Musa sapientum*), Arnstein, Cook & Lacey (82) isolated musarin from a culture which had been obtained by Meredith from certain Jamaican soils. Preparations were obtained which inhibited the banana parasite, *Fusarium oxysporum* var. *cubense*, at about 10 mg. per l. and which were believed to be substantially pure. It was reported to be active against several fungi which are pathogenic to plants but was found to be somewhat less effective against bacteria.

Musarin was reported to be a dextrorotatory acid with an equivalent weight of 4,000 to 8,000, as indicated by potentiometric titrations. The titration curves were complex, presumably due to the presence of other

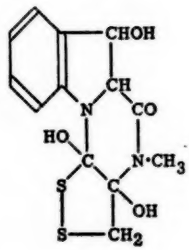
groups. The sodium and potassium salts were found to be soluble in water and in the lower alcohols, but the free acid was insoluble in water. The reaction of musarin with an ether solution of diazomethane led to the formation of an inactive product. Qualitative tests for carbohydrates, proteins, and steroids were negative. At present, no definite conclusions concerning the chemical nature of musarin can be drawn.

Compounds Containing Carbon, Hydrogen, Oxygen, Nitrogen, and Sulfur

Gliotoxin, $C_{13}H_{14}O_4N_2S_2$ (1).—Elvidge & Spring (83) synthesized a degradation product of gliotoxin and presented evidence that it has the structure XIII rather than that previously assigned [see (1)]. On the basis of this evidence, together with the fact that desthiogliotoxin has one C-methyl group, gliotoxin was assigned formula XIV.



XIII



XIV

Penicillin

Penicillin.—Penicillin (1) continues to be the most widely used antibiotic. During the first 10 months of 1949, 104.4 trillion units of bulk penicillin were certified by the United States Food and Drug Administration for use in various pharmaceutical products (84). This represented approximately 59,000 kg. of the procaine salt, 20,000 kg. of the potassium salt, and 7.67 kg. of the sodium salt. These three salts comprised 99.7 per cent of the total amount. The wholesale price for bulk crystalline potassium salt was reported to be 72 to 92 cents per g. (85).

Isolation.—The preparation of pure *n*-amylpenicillin was reported by Leigh (86). The separation from other penicillins was accomplished by partition chromatography of the N-hexamethyleneimine salts on silica gel, using water as the stationary phase and wet ethyl acetate as the moving phase.

Biosynthesis.—Hockenhull, Ramachandran & Walker (87) have suggested that penicillins are formed by *Penicillium notatum* from the amino acids, L-cysteine, D-β-hydroxyvaline, and a carboxylic acid.

Higuchi & Peterson (88) have studied the effects of various media on the

percentages of different penicillins produced. The results are summarized in TABLE I.

Physical Properties.—The dipole moment of methyl benzylpenicillinate in dioxane has been determined by Kumler, Halverstadt & Alpen (89). Their observed value of 4.73 is believed to be consistent with the accepted structure of penicillin and to remove any support which the previously reported value of eight (90) had given to the modified thiazolidine-oxazolone structure (90, p. 450).

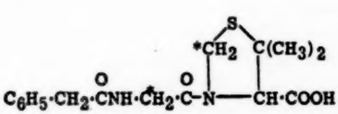
TABLE I

EFFECT OF MEDIA ON THE COMPOSITION OF PENICILLINS PRODUCED BY *Penicillium chrysogenum* Q176

Medium	Percentages of Penicillins Formed			
	Amyl	2-Pentenyl	Heptyl	Benzyl
Synthetic	20	40	30	0
Corn Steep	15	35	20	17
Corn Steep+0.1 per cent Phenylacetic acid	2	15	5	80

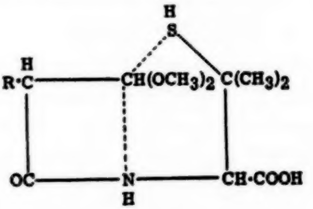
Further evidence has been presented to show that salts of benzylpenicillin give true solutions in water rather than colloidal sols as had been suggested by Hauser, Phillips & Phillips (91). On the basis of conductivity, surface tension, freezing point, and dye solubilization measurements, McBain, Huff & Brady (92) concluded that the sodium salt of benzylpenicillin is an ordinary electrolyte at concentrations below 0.25 *M*. From measurements of the osmotic activities, Lund & Pedersen-Bjergaard (93) concluded that the sodium salt of benzylpenicillin and also the sodium salts of 2-pentenylpenicillin, *n*-heptylpenicillin, and *p*-hydroxybenzylpenicillin, form true solutions in water.

Attempted syntheses.—The synthesis of 3-phenaceturyl-5,5-dimethylthiazolidine-4-carboxylic acid XV was reported by Süs (94). Its methyl ester was prepared by treating a suspension of phenaceturic acid and the methyl ester of 5,5-dimethylthiazolidine-4-carboxylic acid with phosphorus trichloride. The ester was saponified to give the free acid XV which, by removal of hydrogens from the carbon atoms marked by asterisks, would theoretically give benzylpenicillin. However, no attempts to close the β-lactam ring were reported.



XV

Baker & Ollis (95) have attempted to synthesize penicillin homologues in which the C-formylglycine part of the molecule was replaced by C-α-formyl-β-aminobutyric acid or C-α-formyl-β-aminoisovaleric acid residues. Attempted ring closure of compounds XVI and XVII failed to give products with antibacterial activity.



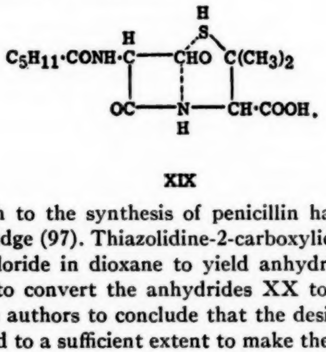
XVI $R = C_6H_5 \cdot CONH \cdot CH(CH_3)-$

XVII $R = C_6H_5 \cdot CH_2 \cdot CONH \cdot CH(CH_3)-$

XVIII $R = C_6H_5 \cdot CH_2 \cdot CONH-$

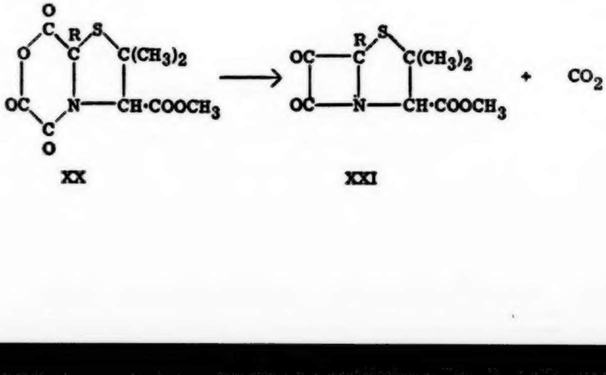
Several unsuccessful attempts to apply this double ring closure to the synthesis of benzylpenicillin from XVIII were recorded in the war-time cooperative work on penicillin (90, p. 855).

Experiments directed toward a similar type of double ring closure have been reported by Cornforth & Huang (96), who attempted to prepare the free aldehyde, N-(amylpenaldyl)-penicillamine XIX, rather than the acetal. The projected ring closure would involve the loss of a molecule of water with formation of the bonds indicated by the dotted lines. Several unsuccessful attempts to prepare XIX were reported.



XIX

Another approach to the synthesis of penicillin has been recorded by Bentley, Cook & Elvidge (97). Thiazolidine-2-carboxylic acids were found to react with oxalyl chloride in dioxane to yield anhydrides (98). However, numerous attempts to convert the anhydrides XX to the desired keto-β-lactams XXI led the authors to conclude that the desired ring contraction was not being effected to a sufficient extent to make the reaction potentially useful.



XX XXI

Further studies on the thiazoline approach (90, p. 857) to the synthesis of penicillin have been reported. Cook & Elvidge (99) have attempted to prepare the required intermediates from acylamidomalonic and acylamidocyanoacetic esters.

Derivatives.—A series of crystalline penicillin amides has been prepared by Holysz & Stavely (100) from butylamine, diethylamine, piperidine, *p*-aminobenzoic acid, and *p*-aminobenzenesulfonamide by reaction with penicillin anhydride. Anhydrous hydrazine was reported to give a N,N′-*bis*-penicillin amide. Hydroxylamine hydrochloride, in the presence of triethylamine, gave the O,N-*bis*-derivative of hydroxylamine. In the standard penicillin bioassay, all of these derivatives were found to be less active than the unsubstituted amide of benzylpenicillin.

The hexamminecobalt^{+3} salt of benzylpenicillin has been prepared by Shortridge & Carr (101) in the form of a crystalline chloroform addition compound. Assays indicated that the cobalt did not increase the *in vitro* activity of the penicillin.

Analytical.—An isotope dilution method for the determination of benzylpenicillin has been developed by Craig, Tindall & Senkus (102). The labeled potassium salt of benzylpenicillin was prepared by the use of phenylacetamide containing excess C^{13} in the amide group as the precursor in the fermentation.

For the determination of benzylpenicillin in beers, a colorimetric method has been described by Boxer & Everett (103). This method is based on the Kapeller-Adler reaction for the determination of phenylalanine. It is not applicable in the presence of other aromatic penicillins (104). The N-ethylpiperidine method for the determination of benzylpenicillin has been modified by Levy, Fergus & Caldas (105) to make it applicable to beers.

A modified spectrophotometric method for determining benzylpenicillin was reported by Levy, Shaw, Parkinson & Fergus (106). Optical densities were determined at 263 and 280 mμ., and the base-line technique was applied.

The determination of amyl, 2-pentenyl, heptyl, and benzyl penicillins in crude mixtures and in beers has been accomplished by Higuchi & Peterson (88). The mixtures of penicillins were hydrolyzed with alkali, and the resulting acids (caproic, 3-hexenoic, caprylic, and phenylacetic) were dissolved in benzene and separated by partition chromatography. Since 3-hexenoic acid underwent partial isomerization and destruction, it was necessary to apply a corrective factor in determining 2-pentenylpenicillin.

An infarred assay for the procaine salt of benzylpenicillin in which the intensity of the β-lactam band at 5.6 μ. was determined has been published by Coy, Sabo & Keeler (107). A new volumetric method for the determination of total penicillins has been developed by Hiscox (108). Potassium ferricyanide was used as the oxidizing agent and ceric sulfate as the volumetric standard. The method is applicable to high potency samples only, since penicillin degradation products act as interfering substances.

Methods of improving the stability of the color that is produced in the

hydroxylamine method for total penicillins have been reported by Boxer & Everett (103) and by Henstock (109).

Kluener (110) has developed a chromatographic method for the quantitative estimation of the different penicillins in beers. The penicillin mixture was applied to a paper strip that had been impregnated with phosphate buffer, developed with ether, and placed on agar seeded with *Staphylococcus aureus*. The results were calculated from the positions and sizes of the inhibition zones. The method is not applicable to mixtures of penicillins having approximately the same distribution coefficients between ether and the phosphate buffer, e.g., benzylpenicillin and allylmercaptomethylpenicillin (111).

Biosynthetic penicillins.[3]—Behrens & Kingkade (112) determined the rate of inactivation by the enzyme penicillinase for 18 biosynthetic penicillins. The most rapidly destroyed penicillin, phenoxymethylpenicillin, was found to be destroyed 1.4 times as rapidly as benzylpenicillin. Cyclopentylmethylpenicillin proved to be the most resistant to the enzyme and was destroyed 0.8 times as rapidly as benzylpenicillin.

When a series of 17 biosynthetic penicillins was assayed against a benzylpenicillin-resistant strain of *S. aureus* (112), the relative effectiveness, compared with benzylpenicillin, varied from 1.7 for 2-thiophenemethyl penicillin to 0.2 for 2-phenoxyethylmercaptomethylpenicillin. Strangely enough, benzylpenicillin was one of the most effective penicillins in preventing the growth of organisms whose resistance was induced by subculturing in the presence of benzylpenicillin.

Rose, Harris, Behrens & Chen (113) have found that allylmercaptomethylpenicillin and butylmercaptomethylpenicillin are much less toxic than benzylpenicillin when applied directly to the central nervous systems of mice, rabbits, and dogs. Furthermore, the authors reported that these new penicillins did not cause the sensitization in guinea pigs which was obtained with benzylpenicillin. Clinical studies have demonstrated that allylmercaptomethylpenicillin is well tolerated by patients who have become sensitive to benzylpenicillin (114).

Penicillin impurities.—A bioassay method for the endotoxin protection factor of crude penicillin has been reported by Anderson & Brodersen (115). It is based on the observation that mice which were given bacterial endotoxins were unable to maintain their body temperature and that the degree of hypothermia was a function of the amount of impure penicillin that was administered. Hobby *et al.* (116) have studied the effect of various penicillin degradation products when used in combination with benzylpenicillin for the protection of mice against *Streptoccus pyogenes* infections. The only compounds which enhanced the action of the benzylpenicillin were *p*-hydroxyphenylacetic acid, caprylic acid, penillic acid, and penicilloic acid; and these gave only slight enhancement. Cram & Tischler (117) have reported

[3] By biosynthetic penicillins we mean penicillins other than amyl, 2-pentenyl, heptyl, or benzyl. Strictly speaking, benzylpenicillin is also biosynthetic (see Table I).

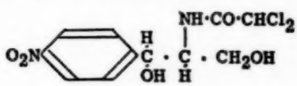
the isolation of nine microbiologically inactive compounds from crude penicillin: tiglic acid, *d*-2-methylbutyric acid, phenylacetic acid, 3-indoleacetic acid, furoic acid, 2-decenedioic acid, and three pigments, one of which was identified as β-penitrin.

Chetomin

Chaetomium cochliodes, $C_{16}H_{17}O_4N_3S_2$, is present in both culture medium and mycelium of *C. cochliodes* (118). Purification of the crude material over a Darco G-60 column yielded a white amorphous product analyzing for $C_{16}H_{17}O_4N_3S_2$. Reduction with zinc dust and acetic acid gave desthiochetomin ($C_{16}H_{19}O_3N_3$). Chetomin gives an alkylindole on zinc dust distillation. It contains a methylimide, and probably a primary amide group. Although it has a very high *in vitro* activity against certain organisms (*S. aureus* inhibited at $1{:}5\times10^8$), it is inactive *in vivo*.

Compounds Containing Organically-Bound Chlorine

Chloramphenicol (*Chloromycetin*[4]), $C_{11}H_{12}O_5N_2Cl_2$ (1).—The structure and synthesis of chloramphenicol XXII have been reported in a series of interesting papers (119 to 122). Chloramphenicol is a neutral compound for which analytical data indicated an empirical formula of $C_{11}H_{12}O_5N_2Cl_2$. A highly important contribution to the structural studies resulted from analysis of the ultraviolet absorption spectrum, which indicated the presence of a *p*-substituted nitrophenyl grouping (119). Chemical tests confirmed the presence of the nitro group. Hydrolysis of chloramphenicol yielded a volatile acid, identified as dichloroacetic acid, and the free base XXIII the structure of which was elucidated by periodate degradation to *p*-nitrobenzaldehyde, ammonia, and formaldehyde. (Chloramphenicol does not react with peri-



XXII

$$p\text{-}O_2N\cdot C_6H_4\cdot \underset{OH}{CH}\cdot CH(NH_2)\cdot CH_2OH$$

XXIII

odate.) The stability of XXIII and the optical activity of the free base and certain of its derivatives indicate that it belongs to the *pseudo*-ephedrine or *threo* series, as shown in XXII [D-(−)*threo*-2-dichloro-acetamido-1-*p*-nitrophenyl-1,3-propanediol]. Chloramphenicol is of unique interest in that it is the first naturally occurring compound known to contain either a nitro or a dichloroacetyl group.

The synthesis of chloramphenicol was achieved by several methods. The base XXIV was prepared by condensing benzaldehyde with nitroethanol and reducing the nitro group catalytically (120). It was also prepared (as the N-acetyl derivative) by condensing formaldehyde with α-

[4] Chloramphenicol is the trivial name for XXII. Chloromycetin is the trademark name of Parke, Davis and Company for this compound.

acetamido-acetophenone and reducing the ketone group catalytically (Raney nickel) (121). Separation of the two racemic forms of XXIV could be readily

$$C_6H_5 \cdot CHOH \cdot CH(NH_2) \cdot CH_2OH$$

XXIV

effected at this point since the desired *threo* form gave an O,N-diacetyl derivative which was less soluble than that of the *erythro* form. The nitro group was then introduced by treating the triacetyl derivative of XXIV with fuming nitric acid. The acetyl groups were removed yielding XXII which was converted to chloramphenicol by heating with methyl dichloroacetate. Of the four optical isomers possible only the natural form had appreciable antibacterial activity. The *erythro* forms were inactive and the (+)-*threo* was only 0.5 per cent as active as the natural (−)-*threo* isomer (120).

Analytical methods for chloramphenicol include a microbiological assay procedure using *Shigella sonnei* as the test organism (123) and a chemical determination (124) based on reduction of the nitro group with titanous chloride and determination of the arylamine produced by the Bratton-Marshall diazotization technique.

Studies on the metabolism of chloramphenicol (125) revealed that in both man and dog it was excreted as such, as a glucuronide conjugation product and as a degradation product. Bacterial enzymes hydrolyze chloramphenicol to dichloroacetic acid and XXIII. It is not attacked by crystalline pepsin or chymotrypsin but is hydrolyzed slowly by papain and trypsin (126). Actively growing bacteria reduce the nitro group to an amine (127). Chloramphenicol does not inhibit the activity of a variety of proteolytic enzymes of bacterial and animal origin (128).

Chloromycetin has rapidly assumed an important clinical role as attested by the number of papers dealing with its pharmacology (129), *in vitro* antibacterial activity (130, 131, 132), and clinical use in the treatment of typhus (133), typhoid fever (134), brucellosis (135), whooping cough (131, 136), early syphilis (137), experimental cholera in mice (138), and other diseases.

Griseofulvin (*Penicillia—several species*), $C_{17}H_{17}O_6Cl$.—Griseofulvin was first isolated by Oxford *et al.* (139), and independently as a "curling factor" (140). Griseofulvin has a marked effect on many fungi, causing stunting, excessive branching, and other effects. It has some toxicity for certain plants but has no effect on bacteria (141).

Aureomycin (1).—Only one preliminary note has appeared on the chemistry of aureomycin. Broschard *et al.* (142) reported that aureomycin was a golden-colored, weakly basic compound, which formed a monohydrochloride. The free base contains nonionic chlorine (*Anal.*, C 54.56, H 5.34, N 5.77, Cl 7.16), is optically active, $[\alpha]_D = -275°$ (methanol), and only slightly soluble in water. The hydrochloride is only moderately water-soluble. In

0.1 *N* hydrochloric acid, aureomycin shows absorption maxima at 230, 262, and 375 mμ.

Aureomycin is unstable in alkali and develops a fluorescence of increasing intensity on alkaline degradation. This property has been applied as the basis for a chemical determination of aureomycin (143). Microbiological assay methods for aureomycin also have been reported (144, 145, 146).

The *in vitro* effectiveness of aureomycin was mentioned in a previous review (1). During the past year a number of papers have described the clinical effectiveness of aureomycin in the treatment of bacterial, rickettsial, and viral diseases (131, 147 to 151). Of particular interest is the action of aureomycin on the viruses responsible for lymphogranuloma inguinale, psittacosis, and primary atypical pneumonia (147, 148, 150). These results are encouraging as far as chemotherapy of virus infections is concerned, although Herrell (147) pointed out that the first two viruses are large ones intermediate between rickettsiae and other viruses, and that aureomycin is not effective in the treatment of influenza, poliomyelitis, or the common cold.

There are indications that development of bacterial resistance to aureomycin may not be as important as it is in the case of streptomycin. Resistance develops slowly and resistant forms rapidly revert to sensitive ones (132, 151). Certain strains of brucella do not develop resistance to aureomycin (or bacitracin) but readily become resistant to streptomycin (151). Strains of β-hemolytic streptococci resistant to aureomycin showed much less alteration in their enzyme pattern than did those resistant to streptomycin or penicillin (152).

Polypeptides

There has been a continuing interest in antibiotics produced by bacteria (153 to 158), despite the fact that the majority of these are peptides which have shown little chemotherapeutical promise. One of the difficulties encountered in dealing with the peptide antibiotics has been the lack of adequate means of achieving or establishing homogeneity. Some promise in this direction has been afforded by the use of counter-current distribution methods. By this technique crystalline gramicidin has been separated into two pure and two impure fractions (159); a subtilin sample thought to be pure on the basis of electrophoretic analysis was shown to be only about 90 per cent pure (160); and commercial bacitracin has been purified to an apparently homogeneous material (161). These results afford promise of further advances in the purification and characterization of the polypeptide antibiotics.

Bacitracin (1).—Craig *et al.* (161) have fractionated bacitracin by counter-current distribution. Despite the inherent instability (161, 162) of bacitracin, a fraction was obtained showing crystallinity and behaving as a homogeneous material in distribution studies. This material on hydrolysis with 6 *N* hydrochloric acid yielded a variety of peptides and the following amino acids: L-histidine, partially racemic L-leucine, L-cystine, L-glutamic acid, DL-aspartic acid, DL-phenylalanine, partially racemic D-isoleucine. Ad-

ministration of bacitracin caused renal damage in humans (163) and in mice (164).

Circulin (*Bacillus circulans*).—McLeod (165) and Murray *et al.* (166) submitted papers almost simultaneously describing antibiotic substances from a *Bacillus* resembling *B. circulans*. The name "circulin" was used for each of the compounds. It was agreed to retain the term for Murray's substance since the organism producing it proved to be *B. circulans*. The second substance was renamed polypeptin (167).

The production, isolation, antibacterial activity, and chemical behavior of circulin were described by Murray *et al.* (166). Circulin resembled polymyxin in its chemical properties but differed in antibiotic spectrum. Circulin is more active against gram negative than gram positive bacteria.

Degradation studies on purified circulin (168) showed it to consist of D-leucine (1 mole), L-threonine (1 mole), L-α,γ-diaminobutyric acid (5 moles), and the nine carbon acid present in the polymyxins. Thus, circulin has the same constituents as polymyxins A and E, but differs from B, C, and D. Polymyxin A was not inactivated by crude trypsin, whereas circulin was. Since no polymyxin E was available for comparison, the possible identity of circulin and polymyxin E remains to be determined. Circulin has no free carboxyl group and all of its free amino groups are contributed by α,γ-diaminobutyric acid. Circulin is inactivated by lipase and by crude trypsin but not by crystalline trypsin. It seems probable that the effect of crude trypsin is due to the presence of a lipase.

In the course of the degradation work on circulin (168) a powdered cellulose column was found to afford separations of amino acids on a column basis paralleling those observed on paper chromatograms.

Gramicidin (1).—Gregory & Craig (159) subjected crystalline gramicidin to a 100-transfer, alternate withdrawal fractionation using a system containing water, methanol, chloroform, and benzene. The distribution pattern indicated the presence of at least four substances. The major component (designated as gramicidin A) was obtained as thin leaves melting sharply at 227 to 228°. A second component (gramicidin B) crystallized as rods melting sharply at 258 to 259°. The remaining fractions were mixtures. Each of the fractions gave similar amino acid analyses, although certain quantitative differences were noted.

Synge (169) has reported fractionation studies on gramicidin[5] hydrolysates. The following peptides were identified in various fractions: L-valylglycine, L-alanyl-D-valine, L-alanyl-D-leucine, D-leucylglycine. Hinman, Caron & Christensen (170) have established, by synthesis of the four isomers, that the valylvaline previously isolated by Christensen from gramicidin hydrolysates was a racemic mixture of D-valyl-L-valine and L-valyl-D-valine.

Synge (169) has developed an enzymatic technique for determining the

[5] The crystalline sample employed proved to be a mixture which was found by Craig to contain an unusually high proportion of gramicidin A.

presence of D-amino acids on paper chromatograms, and presented an interesting discussion of the possible effects of racemization in the peptide chain as hydrolysis proceeds on the final configuration of the amino acids obtained. Synge also has discussed possible methods for the separation and identification of peptides, which often are not susceptible to analysis by paper chromatograms. Edwards (171) has studied the ultraviolet absorption of indole derivatives including acetyltryptophane, and indicated that the tryptophane content of gramicidin could be estimated by determination of intensity of absorption at 281 and 291 mμ.

The molecular weights of gramicidin S (1), tyrocidine (1), and gramicidin have been calculated from diffusion constants (172). The results suggest that gramicidin S has the cyclodecapeptide (rather than pentapeptide) structure. Harris & Work (173) have prepared an open chain pentapeptide containing the amino acids present in gramicidin S. The final product and an intermediate tripeptide had weak activity against *S. aureus.* Peptides containing either D- or L-phenylalanine were equally active and the question is raised as to whether the antibacterial activity of the cyclic peptides is related to the presence of D-amino acids.

Licheniformin (1).—A chemically defined medium for the production of licheniformin and an improved bioassay have been described (174).

Mycosubtilin (*B. subtilis 370*).—From the same strain of *B. subtilis* which produces subtilin, Walton & Woodruff (175) have isolated a crystalline antifungal agent, which they named mycosubtilin. The crystalline product (m.p. 256 to 257°) contained carbon, hydrogen, nitrogen but no sulfur. On hydrolysis it yields ammonia, aspartic acid, tyrosine, and proline.

Polymyxin (*Aerosporin*) (1).—The history, nomenclature, chemistry, pharmacology, *in vitro* and *in vivo* antibacterial activity have been thoroughly reviewed (1, 176). Further studies of polymyxin B have shown (paper chromatogram) that it contains a minor component which can be removed by precipitating polymyxin free base (177). Naphthalene-β-sulfonic acid gives crystalline salts with polymyxin B (177) and E (177, 178) which are useful in the purification of these antibiotics. The optically active C_9 acid present in each of the polymyxins has been shown to be (+)-6-methyloctanoic acid by degradation to the known (+)-3-methylpentanoic acid (178).

Polymyxins B and E have proved to be clinically effective in a series of patients with urinary tract infections (179). Drug resistance did not appear in any cultures from these patients. However, certain reversible neurotoxic symptoms were produced, and when the daily dose exceeded 2.5 mg. per kg., nephrotoxicity was encountered.

Subtilin (1). —The insolubility of subtilin in dilute salt solutions and its failure to be absorbed from animal tissues has interfered with its use in treating animal infections (180, 181). Attempts to overcome this difficulty by use of more soluble esters (160) proved ineffective (180). Dispersing agents (sorbitan mono-oleate) improve the *in vitro* activity (182). More promising is the report that a fraction of subtilin insoluble in serum is removed by precipitation with pectin. The soluble portion was then soluble

in serum in all proportions and gave very satisfactory results in treatment of tuberculosis in guinea pigs (181).

Miscellaneous.—A number of incompletely characterized polypeptide or protein antibiotics have been described during the past year. These are listed below: *Alvein* (*B. alvei* 642) (183), *Laterosporin A and B* (*B. laterosporus*) (184), *Antibiotic XG* (*B. subtilis*) (185), *S. aureus* antibiotic (186), *Polypeptin* (*B. krzemieniewski* M-14) (167, 187), *Bacilysin* (*B. subtilis* A-14) (158).

Antibiotics Not Completely Characterized

Alternaric acid (*Alternaria solani*).—The fungus which produces this antibiotic is normally parasitic on potato and tomato plants. Brian *et al.* (188) have reported that alternaric acid has very little antibacterial activity and that its antifungal activity is very specific. Certain fungi were inhibited at 0.1 to 1.0 mg. per l., while others were unaffected by concentrations as high as 200 mg. per l. Alternaric acid is a crystalline, optically inactive dibasic acid (m.p. 134°).

Biocerin (*Bacillus cereus*).—Biocerin was obtained as an ether-soluble oil by extraction of the culture fluid from a strain of *B. cereus* (189). It is active against both gram positive and gram negative organisms.

Mycomycin (*Nocardia acidophilus n. sp.*).—Mycomycin is obtained in purified form as a stable sodium salt active against a wide variety of bacteria including the mycobacteria (190). It is also inhibitory for yeasts and molds.

Nocardin (*Nocardia coeliaca*).—Extraction of the mycelium of *Nocardia coeliaca* yielded a tuberculostatic agent, nocardin, which was effective in suppressing development of tuberculous infection in mice (191).

Streptocin (*S. griseus*).—The mycelium of *S. griseus* contains an ether-extractable material which is active against gram positive bacteria and trichomonads (192). The material was purified by sublimation.

Antiphage agents.—Two groups of workers have isolated materials from *Aspergillus* sp. fermentation media which inhibit streptophage or staphylophage (193, 194).

Miscellaneous.—Antibiotic substances have also been obtained from lichens (195, 196), from Inoloma [Inolomin from *Inoloma traganum* (197)] and from *Micrococcus*, Micrococcin (198).

Mode of Action

Streptomycin.—The polybasic nature of streptomycin has afforded the basis for speculation concerning its antibacterial activity. It has been observed by several workers that nucleic acids (both ribose and desoxyribose) form precipitates with streptomycin (199 to 202). Cohen (202) found that an *E. coli* bacteriophage (T_2-F), which contains 40 per cent desoxyribosenucleic acid, was precipitated by streptomycin. Massart (203) and Albert & Goldacre (204) have discussed the nature of action of the basic antibacterial substances advancing the general idea that they compete for negative structures of the microorganisms. Rybak *et al.* (200) claimed that the so-called

"streptomycinase" from enterococci filtrates (205) is not an enzyme-inactivating streptomycin, but a nucleoprotein which precipitates streptomycin. In a subsequent paper Gros *et al.* (201) reported that streptomycin agglutinates certain bacteria by formation of insoluble complexes with ribose nucleic acids in the periphery of the cell.

There have been some interesting reports on the effect of streptomycin on enzyme systems in bacteria. Henry *et al.* (206) found that the oxidation of glucose and pyruvate by *S. aureus* (either resting or multiplying cells) was inhibited by streptomycin.

Geiger (207) observed that oxidation of serine by *E. coli* was stimulated by previous oxidation of fumarate and that streptomycin prevented this effect. Umbreit (208) has examined this behavior in detail using threonine instead of serine. The oxidation of threonine by the Gratia strain of *E. coli* was stimulated by previous oxidation of fumarate. Streptomycin prevented the stimulation by inhibiting the terminal stages of oxidation of fumarate (pyruvate oxidation was inhibited also). Umbreit postulated that streptomycin exerts its effect close to the pyruvate-oxaloacetate condensation reaction.

Zeller *et al.* (209) found that streptomycin inhibited the diamine oxidase of *M. smegmatis*, but did not affect diamine oxidase of animal origin. Paine & Lipmann (210) found that neither soybean monophosphoinositide nor brain diphosphoinositide inhibited the antibacterial action of streptomycin. The growth of certain fungi, never previously exposed to streptomycin, is stimulated by its addition to the medium (2.5 mg. per ml. optimum).

Penicillin.—The interesting studies of Maass & Johnson (211) on the binding of penicillin by bacteria (investigated by use of radioactive penicillin) has opened a new approach to the study of penicillin action. These authors find that the rate of binding of penicillin varies with the concentration but eventually reaches a remarkably constant value (0.8 unit per ml. of cells). On the basis of extensive studies with both resting and actively growing cells they postulated that penicillin combines irreversibly with a cell component which is necessary for cell division (but not for respiration or synthesis of cell components). The lowest bacteriostatic concentration of penicillin is the level at which penicillin binding is slightly more rapid than synthesis of the component essential for division.

Eagle & Musselman (212) reported that the bacteriostatic action of penicillin affects all organisms of a population at the same rate and to the same degree. When penicillin is removed, all the organisms recover at the same rate. Death of the cells begins only after multiplication ceases and is an extended process not affecting all organisms simultaneously.

Further studies have been reported on the relation of penicillin to glutamic acid assimilation by *S. aureus*. In penicillin-sensitive strains glutamic acid cannot be concentrated in the cell. Penicillin-resistant strains change their morphology to gram negative and acquire the ability to grow in amino acid-deficient media. Strains of *S. aureus* adapted to growth in amino acid deficient media acquire considerable resistance to penicillin. On the basis of

these observations Gale & Rodwell (213) postulated that penicillin may exert its effect by inhibiting the formation of a substance essential for assimilation of amino acids.

That this is not the only effect of penicillin is indicated by the results of Hunter & Baker (214) who discovered a strain of *B. subtilis* which was sensitive to penicillin and yet would grow on an amino acid-deficient medium.

Mitchell (215) has shown that penicillin causes a disturbance in the nucleotide-nucleic acid balance, which is accompanied by a change in the composition of the nucleotide fraction. Pratt & Dufrenoy (216) presented evidence based on staining techniques that penicillin interferes with enzyme systems involved in hydrogen transport and in the dephosphorylation of ribose nucleic acid. Krampitz (217) also reported that penicillin inhibited the enzymatic decomposition of ribose nucleic acid which otherwise occurs in resting cells of *S. aureus.*

Antibacterial Substances from the Higher Plants

Strictly speaking, substances isolated from the higher plants should not be included in a chapter on antibiotics. However, since there has been considerable interest in such substances they will be listed and, in some cases, discussed briefly.

Allicin,[6] *(Allium sativum),* $C_6H_{10}OS_2$.—Cavallito & Bailey (218) isolated from oil of garlic the antibacterial substance allicin, whose structure was subsequently established as XXV (219). It was subsequently discovered that allicin is not present as such in fresh garlic but is formed by an enzymatic reaction from a heat-stable precursor (220). Stoll & Seebeck (221) isolated

$$CH_2{:}CH{\cdot}CH_2{\cdot}S{\cdot}\underset{\substack{\downarrow\\ O}}{S}{\cdot}CH_2{\cdot}CH{:}CH_2$$

XXV

the precursor and identified it as a new sulfur-containing amino acid, alliin, XXVI (S-allyl-L-cysteinesulfoxide). The enzyme allinase, present in garlic, converts alliin into allicin, ammonia, and pyruvic acid (222). Allinase has no action on cystine or cysteine, but does decompose a series of S-alkyl-L-cysteinesulfoxides (223).

$$CH_2{:}CH{\cdot}CH_2{\cdot}SO{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$$

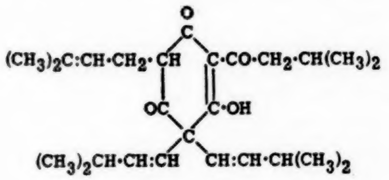
XXVI

Radish seeds are reported to yield an oil with antibacterial properties (raphanin) (224), which is formed enzymatically from a water-soluble

[6] Cavallito *et al.* (220) have proposed that the name allicin be dropped in view of possible confusion with names of certain medicinal products.

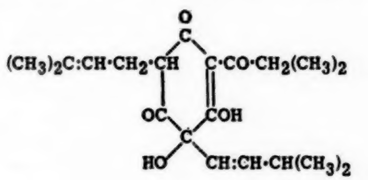
precursor. A survey of seeds of the family *Cruciferae* showed that those containing the enzyme myrosin gave antibacterial products. [Myrosin is the enzyme which hydrolyzes the thioglycoside sinigrin found in mustard (225).] The relationship of these results to the work of Stoll & Seebeck is not clear.

Lupulon (*Humulus lupulus*) $C_{26}H_{38}O_4$.—The fact that lupulon XXVII and the closely related compound humulon XXVIII are the antibacterial agents of hops has been recognized for many years. Lupulon is apparently considered to be more important than humulon and has been reported to be 10 times as active against *S. aureus* (226). Their structures were known as



XXVII

early as 1925, mainly through the work of Wöllmer (227) and of Wieland (228). The *in vitro* activity of lupulon against 12 species of fungi was determined recently by Michener *et al.* (229), who found that concentrations ranging from 40 to 1,600 mg. per l. were required to give 50 per cent inhibition



XXVIII

of the organisms. Chin *et al.* (230) have reported that lupulon was active against the H37Rv strain of *Mycobacterium tuberculosis* in mice when administered orally or intramuscularly, but the latter method of administration was reported to cause renal damage. Salle *et al.* (231) found that the *in vitro* activity of lupulon against *M. tuberculosis* was inhibited by the presence of 10 per cent of horse serum and that the antibiotic was slowly inactivated in the presence of air. Michener & Andersen (226) found that the stability of dilute aqueous solutions of lupulon and humulon was improved by the addition of ascorbic acid.

Rhein (*Cassic acid*) (*Cassia reticulata*), $C_{15}H_8O_6$.—An antibacterial substance isolated from the leaves of *C. reticulata* was named "cassic acid." Anchel (232) has shown that this substance is identical with "rhein" from

rhubarb which has the structure 4,5-dihydroxyanthraquinone-2-carboxylic acid.

Tomatine (*Lycopersicon pimpinellifolium*).—Extracts of tomato leaves exhibit both antifungal and antibacterial activity. The crude extract was termed tomatin (233). A crystalline fraction, tomatine, has been isolated from the crude material and characterized as a glycosidal alkaloid (234). The nitrogen-containing aglycone—tomatidine—was isolated and characterized. Tomatine has antifungal but no antibacterial activity. Tests of several alkaloids suggest that good antifungal agents may be found among quinoline derivatives and other nitrogen heterocycles.

Centaurea maculosa.—Aqueous extracts of the knotted knapweed (*C. maculosa*) yielded a neutral, colorless, bitter compound with activity against both gram negative and gram positive bacteria (235). This substance belongs to the group of unsaturated lactones several of which have antibiotic activity. In comparing the antibacterial activity of this substance with other antibiotics the generalization is made that the nonionic antibiotics which are more soluble in lipophylic solvents will be active predominantly against gram positive organisms, while those which are moderately water-soluble (more than 0.5 per cent) will be active on both gram positive and gram negative organisms.

Antibacterial substances have also been isolated from *Chlorophora excelsa*, wood, Chlorophorin, $C_{25}H_{30}O_4$ (236), from *Curcuma tinctoria*, roots, Curcumin, $C_{21}H_{20}O_6$ (237), from the Indian carrot (238), bananas (239), sweet potato plants (240), ragweed (241), and seed plants (242).

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PARTITION CHROMATOGRAPHY

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Numerous reviews have by now appeared on this subject, and perhaps in future, to avoid repetition, they should be restricted to some limited field or period of time. This present review is intended to cover work omitted from, or subsequent to, the author's review (174) in the *Annual Reports of the Chemical Society* for 1948. General reviews have appeared on chromatography (37, 61, 104, 219, 235, 261, 262) and Hais & Rabek have reviewed paper chromatography as such (118). Reviews on food analysis (194) and protein structure (73) also discuss its use. The *Biochemical Society Symposium* No. 3 on "Partition Chromatography" contains a valuable collection of papers and a bibliography up to the end of 1948. The Faraday Society discussion on chromatographic analysis should also be referred to by those interested in this field (54, see also 55).

Theory of chromatography.—The theory of the development of the chromatogram is of course independent of the mechanisms which determine the distribution of the substances between its moving and stationary parts. The problem of separating two or more substances has been discussed by Glueckauf (108, 109) and others (192, 231) and frontal analysis and displacement development by Claesson (57). Levi (160, 161) and Martin (175) have discussed displacement development on partition columns, the latter in some detail. It is convenient to mention here two experimental papers; Hagdahl (115) has shown that with frontal analysis and displacement development a series of filters of diminishing size connected by narrow tubes greatly improves the sharpness of separation, the irregularities of the front in one section being more or less corrected in the succeeding section. Drake (77) has studied how various methods of packing and various shapes of chromatogram affect the sharpness of the fronts. The superiority of long narrow chromatograms and of low pressure heads is shown.

Rutter (223) has suggested that the streaming potential generated in a chromatogram may exert a noticeable effect upon the movement of some constitutents. The potentials are in fact too small to do this. On the other hand, the zeta potential of the solid, the basis of the streaming potential, may significantly affect the distribution of a charged solute between the phases.

The area of a spot on a paper chromatogram has been calculated by Brimley, assuming that simple diffusion is the sole cause of spreading (35). The application of this calculation to the spot area method of quantitative estimation has been criticised by Fisher, Parsons & Holmes (95). Meinhard (176) has classified chromatograms into different groups, according to the

type of force determining the distribution of the solute between the moving and stationary parts.

Is partition chromatography properly so called?—The term partition chromatography was introduced by Lester Smith to describe the method of separation upon columns of water-laden precipitated silica using a solvent immiscible with water. These columns, originally used by Martin & Synge for the separation of acetyl amino acids, have been used for various separations, and many modifications have since been made. The precipitated silica has been replaced by cellulose, starch, kieselguhr, rubber etc.; the water by buffers, acids, bases and organic solvents; the water-immiscible solvent by a wide variety of solvents, pure, mixed and miscible with the main component of the stationary phase. The term partition chromatography has been objected to on three grounds: (*a*) that in an unstirred system attainment of equilibrium would be too slow if partition between liquids was occurring (12, 62); (*b*) that it is not a chromatogram, a name which should be reserved for columns which utilise adsorption (257); (*c*) that except possibly in a few cases, e.g., kieselguhr columns with immiscible solvents, adsorption always occurs and the systems are indistinguishable from the classical Tswett columns and should simply be called chromatograms (42, 120, 174, 183). The writer disagrees with all of these views (cf. 158, 238). As for the first objection, no evidence has been brought forward in support of this thesis, and calculations on the basis of known diffusion constants (cf. 109) show efficiencies of the right order. Until quantitative evidence to the contrary has been produced this objection should be dismissed. As for the second objection, the essence of the chromatogram is the uniform percolation of a fluid through a column of more or less finely divided substance, which selectively retards, by whatever means, certain components of the fluid.

The third objection should be considered in detail. The basis for the suggestion of partition between liquid phases rather than adsorption on solid surfaces, on or in silica gel, starch or cellulose columns, is that the partition coefficients calculated from the movements of the zones are in good agreement with observed values, except in certain special cases. The agreement is even better if it is assumed that part only of the stationary phase is available to the solute. This is indeed to be expected from the modern views of the swelling and solutions of gels. An excellent discussion of these is given by Hermans (123). In the following discussion attention will be confined to cellulose, but it should be understood that essentially the same ideas can be applied to all the other substances which are at present in use in partition chromatograms. When dry cellulose is exposed to a moist atmosphere water is taken up. The first water taken up has a high heat of sorption and a high apparent density, and a low velocity of diffusion. The velocity of diffusion has a high temperature coefficient showing that the water molecules are firmly bound. As more water enters the cellulose, the heat of sorption decreases, the apparent density drops to that of free water, and diffusion of water and other molecules increases. The change in diffusion constant should be stressed: rapid diffusion is not characteristic of solids.

The amount of chemically bound water is estimated in native cellulose fibre as 5.9 per cent. The rest of the water is held essentially by the same forces as in liquid water. The water which has entered the fibre is not equally and uniformly distributed. It is concentrated in the amorphous regions, as is shown by a comparison of the x-ray diffraction diagrams of dry and moist cellulose which show no change in the reflections due to the crystalline part, which may constitute 60 per cent of the whole.

In the amorphous part the chains are hydrated, much as a molecule of sulphuric acid is hydrated, and in a state of incipient dissolution. They cannot dissolve completely because they are rigidly tied at intervals in the unhydrated crystalline regions and at so called junction-points (regions of close approach of chains, too small and irregular to be called crystalline).

An association of a solute molecule in the water in the amorphous region with one of the carbohydrate chains is no more reasonably called adsorption than is the association of a base with a sulphuric acid ion in solution. There will be carboxyl groups in the carbohydrate chains, the number depending on the treatment the cellulose has received. In so far as these occur in the amorphous portion their effects are to be considered as partition phenomena, similar to those due to small dissolved acid molecules as in buffered columns.

The concentration of the carbohydrate, considered as a solution in water, is of course high in the amorphous region, and the stationary phase in a cellulose chromatogram should be compared with say a strong solution of glucose, or better of some soluble polysaccharide, rather than water saturated with the organic phase. It should therefore cause no surprise that solvents miscible with water can be used. A strong solution of glucose will form two phases with aqueous propanol, the carbohydrate-rich phase containing a relatively higher proportion of water, the other phase a relatively higher of organic solvent. It was suggested by Consden, Gordon & Martin that this process was analogous to salting out, a term which was perhaps too suggestive of ionic interactions.

These effects of the strong carbohydrate solutions will also operate on solute molecules and not only on the solvent. Thus if we consider a starch or cellulose column with water as the mobile phase, it is to be expected that the solubility of the long chain monocarboxylic monoamino acids in the carbohydrate solution will be less than that of short chain acids. Thus the separation of glycine and alanine by Moore & Stein on a starch column with water does not prove adsorption by the starch. It is equally explicable as a "negative" partition effect, the alanine being less relatively soluble in the carbohydrate solution than the glycine, both being probably less soluble than in pure water. It should be remembered that if solubility in free water and in the water within the starch were identical the acids would not move with the front of the moving phase in the column, but at a lesser rate governed by the relative cross sections of moving and stationary solvent. A retardation is thus no proof of adsorption.

Basic amino aicds are comparatively strongly retarded by starch or cellulose when the solvent is water. This may be due to the effect of carboxyl

groups in the carbohydrate or to adsorption on the crystalline part of the cellulose, or to association with the dissolved carbohydrate chains. Tryptophane may be adsorbed or may associate with the dissolved chains. A test of the relative solubilities of tryptophane and leucine in water and various carbohydrate solutions might settle this matter. The fact that tryptophane is so strongly adsorbed by charcoal, suggests that adsorption is probable; but acetyltryptophane, which is also strongly adsorbed by charcoal, is little retarded by cellulose. A specific interaction with dissolved carbohydrate may therefore exist.

Adsorption and partition phenomena do of course coexist in the same system. The water-laden silica columns adsorb acetylated amino acids powerfully from pure chloroform or cyclohexane and some alcohol must be present to act as an eluting agent before they will function as predominantly partition columns. Similarly charcoal laden with benzene or oleic acid will function both by adsorption and partition. Cellulose "activated" by nitric acid (42) for the separation of inorganic ions may well have part of its properties attributable to carboxyl groups, though as suggested above, this may still be properly considered as a partition effect.

In sum, the writer feels that not only columns of kieselguhr containing macroscopic droplets of stationary phase, but also columns of gels of any kind which are swollen with solvents may properly be considered as partition chromatograms. This includes, incidentally, all of the commonly used synthetic resin ion exchangers. Apart from these cases where a microscopically porous structure fills with stationary phase, the dividing line as to the existence of partition effects should probably be drawn by considering the swelling of the solid concerned. If the solid has to be swollen by solvent before the solute can diffuse within it, partition phenomena will occur.

The reviewer had previously held the view that it was an academic question of little practical importance whether the phenomena that occurred were called adsorption or partition (and the latter term has been used here in a very restricted sense). He was shaken out of this view, however, by a remark of Moore's, that any agreement between the partition coefficients calculated from starch columns and those measured directly between two liquid phases was "purely coincidental"; that the solutes were adsorbed upon internal surfaces, which were a kind of water-starch complex. (This latter sentence must be regarded only as the writer's interpretation of Dr. Moore's remarks.) With this picture the possibility of predicting the behaviour of untested substances is largely jettisoned since adsorption is far less understood than partition. It would also, as Synge has pointed out, lead to the expectation that an optically active column of starch or cellulose would resolve racemic amino acids (238). This has not yet been observed.

Prediction of the partition coefficient.—Martin (175) has considered the consequences of making the assumption, as a first approximation, that the free energy change associated with moving a given group, e.g. a CH_2 group, from one phase to the other of a given phase pair is independent of the other

groups in the molecule. The total free energy change of the whole molecule is then a simple additive function of its constitutent groups. Thus isomers with the same functional groups would be expected to have identical partition coefficients irrespective of the solvents.

It follows from the assumptions that the addition of a particular group to a molecule will change its partition coefficient in a ratio depending only on the group and the phase pair concerned. Thus the same ratio will relate the partition coefficients of all neighbouring pairs of a homologous series, and this ratio between the pairs will be unaffected by forming derivatives. This latter prediction is contrary to the usual expectation that the addition of a large group of atoms will tend to swamp the differences between molecules.

The rule holds well enough to be of service in forecasting the partition coefficients of peptides, given knowledge of the partition coefficients of the amino acids and a few peptides to evaluate the constants. Bate-Smith & Westall (14) have shown that it holds also for hydroxyl groups and glucose groups in a large number of plant pigments and related substances.

Instead of expressing the results in terms of the partition coefficient they use $R_M(\log 1/R_F - 1)$ which is a simple function of the partition coefficient and the constants of the chromatogram, so that only the R_F needs to be measured. On plotting R_M against number of groups, different homologous series form more or less straight lines, practically parallel for any given group.

Deviations from expected values will occur from two principal sources. Firstly, the rest of the molecule will influence the substituent group; a rather extreme example is that a phenolic hydroxyl is noticeably different from an aliphatic hydroxyl. Secondly, for steric reasons the solvent molecules may be unable to approach near enough in certain cases to show the expected energy of bonding. Thus with long chain compounds the difference resulting from a single CH_2 is less than with short chains, which may well be the result of the long chain coiling into a ball in the polar phase, which would reduce the change in surface to roughly the two-thirds power of the change in a straight molecule.

It might be expected that steric factors would be so much more important in adsorption on a solid surface that adsorption would better distinguish between isomers than partition; it is highly improbable that the separations that can be effected by classical chromatography in the carotenoids could be duplicated by partition chromatography.

Chromatographic Techniques

Methods of irrigating paper chromatograms.—Most of the possible ways of inducing liquid to flow through paper seem now to have been tried (174). The paper may hang from a trough and the liquid be allowed to syphon over and down (265). This method gives rapid development and permits the solvent to travel several times the length of the paper. In this latter case the

bottom of the paper may be cut to a point (172) or have fastened to it, an absorbent pad of wood pulp which speeds development (180). Alternatively the lower end of the paper may dip into the solvent which rises by capillarity (267). Development is slower and the solvent cannot run from the end of the paper, which is sometimes a convenience. The paper may be hung over a bar with the lower end dipping in the solvent, which travels first up and then down, with some gain in rate of development (R. J. Block, private communication). The minimum of apparatus required in these methods is advantageous where large numbers of chromatograms are needed. The large sheets of paper may be rolled into a cylinder, or a spiral (168) and stood in the solvent, and two-dimensional chromatograms can thus be run in a small cylindrical vessel. The small free space is advantageous particularly when volatile solvents are used. Each strip may be done in its own test tube (222).

With complex solvents, buffered papers and very volatile solvents, it is most important that thorough equilibration should occur throughout the system. Hanes & Isherwood, working in a thermostatic enclosure, attain this by keeping the paper in gentle movement by a magnet moving outside the box, a piece of iron being attached to the bottom of the paper (120).

Columns have been made of filterpaper discs by Mitchell & Haskins (182). Several boxes of filter papers are piled together between perforated plates under pressure from a clamp and solvent syphoned to the top, the free surface of the liquid being slightly below the uppermost paper. After development individual papers can be extracted for test, and the papers containing complete zones can, if desired, be placed at the head of a new column.

Other apparatus.—The use of fraction cutters to collect the effluent from the column is now recognised as the most satisfactory method for many purposes. Though many analyses have to be made the additional information obtained makes it worth while. Various arrangements have been recently described (36, 64, 82, 203, 213).

Photoelectric apparatus has been used to measure the area of the spots (94) or intensity of absorption (100, 187).

Lindberg & Hummel have described apparatus for the estimation of radioactivity on paper (163).

Arrangements for evaporating material on paper prior to chromatography have been described (248) and also for washing off after development (270).

Difficult separations.—As would be expected on theoretical grounds, improved separations of very similar substances can be obtained with systems giving low R_F values. Thus leucine and isoleucine can be well separated with amyl alcohol (270) or tertiary amyl alcohol (180) with long periods of development. Similarly with carbohydrates (137).

Accurate R_F values.—Bate Smith (13) has studied the conditions necessary to obtain accurate R_F values. The following recommendations are made: (*a*) the temperature should be controlled to $\pm$ 0.5°C; (*b*) the time of

running should be constant; (*c*) the paper should be equilibrated with the atmosphere in the chamber for 24 hours prior to irrigation with solvent (137); (*d*) one batch of paper should be used for all determinations; (*e*) a control substance should be run on every chromatogram. If the R_F differs from the standard by more than ± 0.02 that run should be discarded, and fresh solvents used; (*f*) when solvents are used which undergo reactions, e.g., esterification, they should be allowed to stand three days at the working temperature prior to use. With these precautions the reproducibility is very high, comparable probably with those obtainable in the starch columns of Moore & Stein (183). With the magnet attached to the paper (120) the time of equilibration can no doubt be reduced. Special purification of phenol has been recommended (78).

Purification of paper.—Purification of the paper is also proving necessary in various cases, and perhaps the time is nearly ripe for an approach to be made to the manufacturers with standard specifications for different purposes. Neutral substances appear to be less affected by impurities than charged ones. Thus Hanes & Isherwood (120) find it necessary to wash the paper with various acids and sometimes with 8-hydroxyquinoline, or to treat with hydrogen sulphide before carbohydrate phosphoric esters will form well shaped spots. These impurities are probably inorganic. Wynn (273) finds that the paper contains peptides which can be washed off with water in 48 hours. Jones (139) finds that paper varies greatly in fluorescence and that with some washed papers fluorescence can no longer be used to reveal the position of amino acid spots.

Use of ultraviolet light.—The application of ultraviolet light to detect substances on paper chromatograms continues. It might be preferable to add fluorescent material to purified paper to show adsorbing materials or quenching of fluorescence, as is done by Brockmann in adsorption columns (38). This has been done after chromatography of inorganic substances by Osborn & Jewsbury (193) and Pollard, McOmie & Elbeth (208) who employ a variety of reagents.

Markham & Smith (171, 172) have obtained excellent prints from chromatograms of purines, pyrimidines etc. using the 2650 Å mercury line. Holiday & Johnson (130) have observed spots of these substances as dark spots against a fluorescent background, presumably because of absorption of the exciting radiation. Phenolic substances, (13, 14, 259), porphyrins (188, 189), pteridines (142), flavin decomposition products (117), anthranilic acid (266), and N substituted amino acids (107) have all been seen as fluorescent or dark spots in ultraviolet light. No corresponding use of infrared radiation seems yet to have been made.

Use of tracer elements.—Keston, Udenfriend & Levy (144) make the I^{131} pipsyl derivatives of a mixture of amino acids and mix them with S^{35} pipsyl derivatives of an amino acid. This mixture is then chromatographed. The radioactivity of I^{131} and S^{35} can be independently estimated and hence the amount of the amino acid in the original mixture can be inferred. Complete

resolution on the chromatogram is not essential provided a region can be found where there is a constant ratio of I^{131} to S^{35}. The method of separation has been further developed (145) solvent extractions being followed with paper chromatograms. Glutamic and aspartic acids, hydroxyproline, serine, threonine, glycine and alanine have been estimated in several proteins. This is clearly a powerful method.

The biosynthesis of thyroxine has been further studied. Taurog, Tong & Chaikoff (242) by use of paper strips, find in the thyroid diiodotyrosine, thyroxine and inorganic iodine after administration of I^{131} to rats. Tishcoff *et al.* (245) on two dimensionals find in addition monoiodotyrosine, diiodothyronine and two unknown iodine compounds. They find also that collidine, while extracting the other substances, fails to extract thyroxine from the gland, and assume it occurs only in the protein.

Calvin & Benson (47, see 18) have studied the path of carbon in photosynthesis. *Chlorella* growing in continuous culture was exposed for varying times to $C^{14}O_2$. Extracts made after 30 seconds exposure showed on autoradiographs of two dimensional chromatograms that the major part of the C^{14} was in phosphoglyceric acid, triose phosphates and hexose phosphates. After 90 seconds the major part was in sucrose, which was the first free carbohydrate formed. The sucrose was formed from fructose-6-phosphate and glucose-1-phospate. Fructose-6-phosphate was formed before the glucose phosphate moiety and after 90 seconds the activity was evenly divided. This method of following successive transformations appears to be a model of elegance and economy of effort. No doubt it can be widely applied.

The phosphate turnover in various nucleotides in respiring liver homogenate in the presence of radioactive orthophosphate has been studied by Hummel & Lindberg (135), using paper chromatography. Flavin-mononucleotide, pyridine nucleotides and adenylic acid showed no activity, but adenosinetriphosphate and flavin-adenine-dinucleotide did. The latter on account of its high specific activity is thought to be intimately associated with aerobic phosphorylation.

The nitrogen turnover in purines from polynucleotides has been studied by Reichard (216) using N^{15}. The purines were separated on starch columns. He finds that guanine in ribonucleic acid from regenerating liver and from desoxyribonucleic acid in intestine is not synthesised via adenine.

The metabolism of urea has been investigated by Leifer, Roth & Hempelmann (153). C^{14} urea was injected into the mice. C^{14} was found in the urine only as urea, and as $C^{14}O_2$ in the breath.

Lester Smith (157), using S^{35} labelled penicillin, has compared results of measurements, after chromatography on paper, by Geiger counter and inhibition zone diameter on bacterial plates; substantial agreement was found.

Amino Acids, Peptides, and Proteins

Reviews of the application of partition chromatography to amino acids and peptides [Jones (139)] to the study of amino acid and protein metabo-

lism [Dent (70)] and to the study of protein structure [Sanger (227)] have recently appeared.

Numerous variations in the solvent mixtures for paper chromatograms are reported but most are mixtures of those that have been used before, [e.g. (31, 41, 180, 201, 270)]. A comparison of various solvents is given by De Verdier & Agren (75). Bentley & Whitehead (19) suggest furfuryl alcohol and tetrahydrofuryl alcohol.

Iodine, applied as vapour or spray, has been used by Brante (31) to reveal the positions of various nitrogenous substances. Fugitive brown or yellow spots are produced, but the substances are not destroyed and other reagents for amino acids or other substances may subsequently be used. It may be valuable to mark spots for quantitative analysis. Crumpler & Dent (63), by dusting the paper with $CuCO_3$, can distinguish α-amino acids from other ninhydrin reacting subsances. Patton & Foreman (201) use *o*-phthalaldehyde to give a green spot, fluorescing chocolate brown with glycine. Synge (236) determines the optical configuration of an amino acid spot by spraying with D-amino acid oxidase. Nicholson (190) prefers to put ninhydrin in the developing solvent rather than spraying later (a method which the reviewer does not recommend).

Quantitative methods.—Quantitative methods (for general discussion see Gordon, 113) for amino acids on paper using ninhydrin and measuring the spot density and area are reported by Bull, Hahn & Baptist (41, cf. 100). Bolling, Sober & Block (25) similarly used diazotised sulphanilic acid for histidine and tyrosine. Fisher and his collaborators (94, 95) have given further details of their spot area method. Landua & Awapara (148) extract the spot, marked with the minimum of ninhydrin, and develop the colour with the Moore-Stein ninhydrin mixture. A final assessment of the value of these and earlier methods (174) must await wider use, but it is already clear that they are well suited for rapid and not too exacting analyses. Fitzpatrick suggests that his spectrophotometric ninhydrin method (96) would be applicable to paper chromatograms.

Moore & Stein (183) have now completed their scheme for amino acid analyses on starch columns. Most of the acids can be resolved on a single column using a butanol, propanol, 0.1 N HCl mixture as solvent. Glutamic acid and alanine require a separate column and the fastest running acids need the column reported earlier. The effluent is collected with the help of their fraction cutter and their colorimetric ninhydrin method is used to estimate the amino acid concentration in each tube. The accuracy is given, conservatively, as $100 \pm 3\%$. It would seem to be the method of choice where a complete analysis of a protein is required.

Pipsyl derivatives have been discussed above (144, 145). Dinitrophenyl (DNP) derivatives [Sanger (227)], though apparently well adapted to quantitative analysis of amino acid mixtures, have been used for this only incidentally (177). Their principal use has of course been for the determination of the free amino groups in proteins and peptides because of the resistance of DNP

derivatives to acid hydrolysis. Buffered silica columns (23, 177, 178) may be used in place of the original water-laden columns (227).

Unusual amino acids.—Ackermann & Kirby (2), using paper chromatography have identified pantonine (α-amino-β,β-dimethyl-γ-hydroxy butyric acid) in *E. coli.* Bergstrom & Paabo (21) have shown that α-amino adipic acid may be separated from glutamic acid on paper using a *n* butyric-*iso*valeric acid-water mixture. α-Amino adipic acid and citrulline will form arginine in the liver (79). Steward, Thompson & Dent (234) have identified γ-amino butyric acid in potato sap (not present in the protein). Work (270) has identified α-amino butyric acid, γ-amino butyric acid and β-alanine, in extracts of *C. diphtheriae* and has shown the presence of unidentified acids in the extract and in hydrolysates of the cell residue.

Gal & Greenberg (107) have studied the separation of alkyl substituted amino acids which may be distinguished under U.V. irradiation from amino acids on the paper chromatograms. Plattner & Nager (206, 207) have also studied these acids and have shown the presence in hydrolysates of the antibiotic Enniatin A of N methyl isoleucine. N methyl amino acids were distinguished from amino acids by the reagent β-nitrobenzoyl chloride. Seven strains of fusaria were examined for these acids.

An unidentified substance reacting with ninhydrin has been isolated from agene (NCl_3) treated protein by Bentley *et al.* (20). A variety of methods was employed, including electrodialysis and water-laden silica columns with phenol as solvent. The crystalline material causes fits in rabbits similar to those produced by agene-treated flour. It gives a single spot on a paper chromatogram.

Hird & Trikojus (125) have studied the formation of thyroxine in artificially iodinated proteins. Thyroxine, diidotyrosine, and 3,3′,5-triiodothyronine were found. The separation of these substances on paper laden with 2*N* NaOH by use of a mixture of amyl and butyl alcohols is described. Pitt-Rivers (204) has studied the formation of thyroxine from diiodotyrosine and demonstrated by paper chromatography the formation of alanine. The biosynthesis of thyroxine has been mentioned above (242, 245).

Amino acids in tissues, excretions, etc.—The application of paper chromatograms to urine, plasma, etc. has been reviewed by Dent (69, 70). Agren & Nilsson (5) have observed 21 compounds in blood plasma; four were not amino acids but gave colour with ninhydrin (cf. 75). Normal urines (119) have been examined by Hall, and shown to contain little or no methionine by Tomich (246). Amino-aciduria has been studied by Ames & Risley in progressive muscular dystrophy (6, 7) and in liver disease by Uzman & Denny-Brown (251) and Young & Homburger (275). Wynn reports the presence in urine of a peptide not reacting with ninhydrin (273). Free amino acids in saliva are reported by Goldberg, Gilda & Tishkoff (110), and those released in semen after ejaculation have been reported upon by Lundquist (167). Gordon (112) has compared the amino acids and peptides in calf-embryo muscle juice with those of adult beef, and reports the presence of

aminoethyl- and hydroxylysine-phosphates. Roberts & Tishkoff (218) have compared the three amino acids present in new-born and adult mouse epidermis before and after treatment with carcinogen, with those in the resulting carcinoma. In the tumour the content is lower and in the hyperplastic tissue higher than in the normal. Awapara (10) has examined the free amino acids of various rat tissues. The free amino acid distribution in the haemolymph of insects has been recorded by Findlayson & Hamer (90) and Agrell (3) has followed their metabolism during metamorphosis.

Dent & Schilling (72) have studied protein digestion in the dog. After feeding casein, casein hydrolysate, beef or human serum proteins, amino acids increased in the blood plasma, suggesting that the proteins were absorbed as free amino acids. Few peptides were apparent. After feeding on dog-plasma proteins no rise in amino acids occurred, suggesting that these proteins were absorbed intact.

Polypeptides and proteins.—Synge (237) has reviewed the naturally occurring peptides and mentioned briefly methods for their investigation. Borsook *et al.* (27) have continued the study of their liver peptides. Similar peptides have been isolated from the livers of six species of mammal and one fish. Guinea pig blood, heart, kidney and spleen also contain such a peptide, which was investigated, after hydrolysis, on starch columns using Moore & Stein's technique (183). The proportions of the various amino acids in the peptides from different animals were similar but not close enough to establish their identity.

A general review on polymixin has been given by Brownlee (40). The chemistry has been described by Jones and his colleagues (49, 138) and Bell *et al.* (17). The latter used kieselguhr columns loaded with sulphosuccinate buffer, with butanol as solvent, for its purification. Sanger's methods were employed to determine free amino groups. They conclude that it is a cyclic polypeptide. Jones (138) shows that the polymixins form a family of related substances, produced in different proportions by different strains, all of which contain threonine, α,γ-diaminobutyric acid and a fatty acid, $C_9H_{18}O_2$. D-leucine, phenylalanine, and serine are variously present in different members of the family. The polymixins form satisfactory spots on paper chromatograms in spite of their relatively high molecular weight.

Lycomarasmin has been analysed by Plattner *et al.* (205) and by Woolley (269); glycine and aspartic acid have been identified in the hydrolysis products and structures are suggested. Amino acids in a strepogenin concentrate are recorded (202), and in liver and casein fractions (75) and in a growth factor for *C. diphtheriae* (53). Mueller & Miller (186) have purified, by Craig's methods (62) and starch columns, a factor in a tryptic digest of casein needed for tetanus toxin production. A study of the fractionation of a pepsin digest of egg albumin on carbon and paper has been made by Moring-Claesson (185).

Peptides from partial hydrolysates of gramicidin have been characterised by Synge (236) who found D-leucylglycine, L-alanyl-D-valine, and L-alanyl-

D-leucine. Synge & Tiselius (239) separated tryptophane peptides on carbon and on paper using various solvents.

A large number of peptides of glutamic and aspartic acids were found in a partial acid hydrolysate of wool by Consden, Gordon & Martin (60), and Consden & Gordon (59) report also many cystine peptides, separation being effected after oxidaton of the cystine peptides to cysteic acid peptides.

Middlebrook has determined the end groups of wool (178), its hydroxylysine content (177), and has studied its reaction with formaldehyde (179).

Sanger has continued his sudies of insulin and has reviewed work on its structure (224). Two fractions were isolated from oxidised insulin (225): fraction A with glycyl end groups and containing no arginine, histidine, glycine, phenylalanine or threonine; fraction B with phenylalanyl end groups and containing all the other amino acids present in insulin. He has also (226) demonstrated species differences in insulin in different mammals in the end groups and the amino acid constitution of fraction A. Butler, Phillips & Stephen (44, 45) report studies on enzyme digests of insulin. Woolley (268) has studied DNP peptides split from DNP insulin and DNP trypsinogen by pancreatin.

The amino acid constituents of serotonin are reported by Rapport (214), of bradykinin by Rocha e Silva *et al.* (221), of Bence-Jones protein by Agren (4) and Dent & Rose (71). The latter, on the basis of lack of methionine and its continuous production in a dying patient, suggest that Bence-Jones protein is a protein associated with a virus which is the cause of multiple myelomatosis. The amino acid composition of tropomyosin has been determined by Bailey (11), of connective tissue proteins by Bowes & Kenten (28) of secretin by Edman & Agren (83) of lysozyme by Fromageot & Privat de Garilhe (103) and of β-lactoglobulin and bovine serum albumin by Moore & Stein (233). The amino acid composition of normal and Brucella immune rabbit serum has been compared by Jones, Pollard & Holtman (140). The composition of end groups of whale myoglobin is reported by Schmid (228) and of salmine by Tristram (247). The amino acid composition of bacterial flagella has been studied by Weibull (258), and of *C. diphtheriae* by Work (270, 271, 272).

Desnuelle & Casal (74) have studied the relative stability of different peptide bonds in protein to acid hydrolysis, using Sanger's method to determine the free amino groups of the peptides produced. Serine and threonine were found to be most labile.

Porter (209) has found that not all the lysine side chains of native proteins will react with dinitrofluorobenzene under Sanger's conditions. After denaturation all will react. Lea & Hannan (149) have shown, using Sanger's methods, that during the reaction between casein and carbohydrates free amino groups disappear.

Various attempts have been made to separate proteins on paper. Tiselius (243, 244) and Shepard & Tiselius (230) have employed aqueous salt solutions to promote the adsorption of protein on paper. There seems to be dif-

ficulty in inducing the proteins to run as a compact band and a tendency to denaturation exists, though with certain dyestuff molecules the method works well. Franklin & Quastel (101), using hemin compounds of the proteins, detecting with benzidine and hydrogen peroxide, and using various aqueous solutions as solvents, obtained some separation. Mitchell, Gordon & Haskins (181) separated adenosine deaminase, amylase and phosphatase in takadiastase on a column of filter papers, using an ammonium sulphate solution of gradually falling concentration. They suggest also the use of solvents of changing pH. It is improbable that partition is playing any part, and even adsorption may be playing a lesser part than simple solubility. Nevertheless it seems to be a most promising field for further work.

Other applications of paper chromatography.—The use of paper chromatograms for routine control of the medium for diphtheria toxin production is described by Linggood & Woiwod (164). The nitrogen metabolism of bacteria has been studied by Proom & Woiwod (210). Paper chromatograms have also been used to control fractionation by ion exchange resins (197, 199, 200), to identify glutamic acid in enzymatic digests of glutamine (33), and aspartic acid in the study of the transformation of fumarate to oxaloacetate (58), and to check the purity of synthetic peptides (121, 156).

Carbohydrates

A general review of the chromatography of sugars has been given by Binkley & Wolfrom (22). The application of partition chromatography to carbohydrates has been reviewed by Partridge (198) and Hirst & Jones (127) (see also 174).

Flood, Hirst & Jones (97) describe the estimation of sugars by Somogyi's reagent after washing the spot from a paper strip. Hirst, Hough & Jones estimated methyl sugars after separation on paper with alkaline iodine. Various starches, glycogen and araban were studied (129). Tagatose was recorded for the first time from a natural source. (128). Hirst & Jones (126) use oxidation with periodate for the estimation of sugars and apply the method to paper strips. Hough, Jones & Wadman (134) describe the separation on columns of powdered cellulose of simple sugars and of methyl sugars. Dyestuffs are used as markers to facilitate cutting.

Jermyn & Isherwood (137) have made a study of optimum conditions for good separations and reproducible results on paper chromatograms. They prefer three component solvents chosen to give low R_F values. They describe a modified quantitative method.

Bell & Palmer (16) described the quantitative separation of di-, tri- and tetra-methyl fructoses on water-laden silica columns. The estimation was by weight and the fractions were shown to be pure by paper chromatograms.

Reagents for paper chromatograms.—Partridge (196) has used aniline hydrogen phthalate as a reagent for sugars on paper chromatograms. Aldopentoses give a red colour and aldohexoses and desoxy sugars a green or brown colour. Pacsu, Mora & Kent (195) spray the chromatogram with

alkaline permanganate; a yellow spot against a purple ground, which must be marked immediately, shows the presence of a sugar or other reducing substance. Horrocks (133) has found that benzidine will show the reducing sugars on paper without reacting to urate, and is particularly suited for urine analysis.

Paper chromatograms have been used to study sugars in urine (80), and in peas (81, 39), the polysaccharides of cell walls (136), and of soil bacteria (99) and of *Penicillium luteum* (102). They have been used also for blood group substances [see review by Morgan (184) and Kabat *et al.* (141)], in a quantitative analysis, accounted for all the carbohydrate of blood group A substance as fucose, galactose and glucosamine. Bray & James (32) find also fucose, galactose and N acetyl glucosamine.

Mapson & Partridge (170) have separated ascorbic acid and related substances on paper using 2,6-dichlorophenolindophenol or alkaline silver nitrate for detection.

Hanes & Isherwood (120) have separated the hexose monophosphates except fructose-1- and fructose-6-phosphates and glucose-6- and mannose-6-phosphates. They have made a wide survey of possible solvents, and find acid or basic solvents are best. Calvin & Benson (47) have published autoradiographs of several carbohydrate phosphates in their photosynthetic study of chlorella with $C^{14}O_2$.

Purines, Pyrimidines, and Related Substances

Vischer & Chargaff (252) separated adenine, guanine, hypoxanthine and xanthine, and uracil, cytosine and thymine on paper strips using a variety of solvents. The substances were detected by treating the strips with a mercury salt, washing and converting to mercuric sulphide. With such guides other chromatograms were extracted and the amounts estimated by ultraviolet absorption. These methods were used to analyse yeast and pancreas ribonucleic acids (253) and desoxypentose nucleic acids from avian tubercle bacillus and from yeast (255), calf thymus and beef spleen (52). The sugar in desoxypentose nucleic acid was identified as 2-desoxyribose.

Markham & Smith (171, 172) have described one and two-dimensional paper chromatograms of purines, pyrimidines and their ribosides. A list of R_F values in seven solvents is given, and their relation to the structure of the molecules is discussed. Their methods of printing the spots on to photographic paper is described above (cf. 130).

Vischer, Magasanik & Chargaff (254) have separated adenylic, guanylic and cytidylic acids from yeast ribonucleic acid, from each other and from muscle adenylic acid on paper, and detected the spots with uranyl salts, and ferrocyanide. Carter & Cohn (48) have separated from yeast, using ion exchange and paper chromatography, two adenylic acids which differ from muscle adenylic acid. The two yeast acids are probably adenosine-2-phosphoric acid and adenosine-3-phosphoric acid.

Daly & Mirsky (66), using a single starch column with a solvent mixture

of *n*-propanol and 0.5 *N* HCl have separated thymine, uracil, cytosine, adenine, guanine and hypoxanthine; ultraviolet absorption is used for quantitative analysis. Edman *et al.* (84) use a starch column with butanol-water-methyl cellosolve mixture to separate adenine and guanine. Reichard (215), using starch columns for separation, obtains good yields of nucleosides from digests of ribomononucleotides with prostate extract.

Work on phosphate (135) and nitrogen turnover (216) has been mentioned in the section on tracer elements (p. 524). Zamenhof & Chargaff (277) have measured the proportions of adenine, guanine, thymine and cytosine in the desoxyribonucleic acid of calf thymus and yeast, before and after digestion with crystalline desoxyribonuclease. Material first split off was similar to the intact acid in composition but a resistant core differed considerably.

Organic Acids

Fatty acids.—The separation of fatty and other acids by partition chromatography has been reviewed by Elsden (86). Ramsey (211) has separated n- and *iso*-butyric acids on water-laden silica with 2,2,4-trimethyl pentane. Ramsey & Patterson (212), using hexane against 2-aminopyridine-furfuryl alcohol on silica, have separated saturated acids from C_{11} to C_{19}. For complete separation a difference of two carbon atoms is needed. Higuchi & Peterson (124), after hydrolysis of penicillin, have separated the side chain acids on kieselguhr (celite) columns with sulphuric acid or with a sulphuric-phosphoric acid mixture as the stationary phase.

McClymont (169) has used buffered silica columns (see 86, 174) to study the utilisation of acetic acid by the mammary gland. Achaya (1) has used Elsden's method (86) in a study of rancid ghee. Boldingh (24) has developed further his reverse phase chromatogram (see 174) and can now separate on a single column of rubber powder, saturated acids from C_6 to C_{24}. Again for complete separation a difference of two carbon atoms is required. Aqueous methanol, with or without chloroform, is the mobile solvent.

Other acids.—Claborn & Patterson (56) separated lactic and succinic acids (also formic and acetic) on a glycerol-laden silica column with butanol-chloroform solvent. Fischbach, Eble & Levine (92) used a silica column in the separation of *o*-hydroxyphenyl acetic acid from crude penicillin, the activity of which it enhances. Gottlieb (114) used a water-laden silica column for the separation of tropic and atropic acid. Marshall, Orten & Smith (173) separated eight different acids on a water-laden silica column with an amyl alcohol-chloroform mixture; they have estimated fumaric acid in animal tissues.

Lugg & Overell (166) separated some 16 dicarboxylic and hydroxy acids on one and two dimensional paper chromatograms. They used solvents containing formic or acetic acid to suppress ionisation [or combine with the acids being separated, (238)] and to give compact spots. Butyl, amyl, and benzyl alcohols; methyl, isobutyl, and diisopropyl ketones; and mesityl oxide were

used as solvents. The spots were detected by spraying the dry papers with indicators.

Fink & Fink (91) also used paper for separating hydroxamic derivatives of volatile or nonvolatile organic acids. Alcohols, ketones, phenol and isobutyric acid were used for development, the latter pair for two dimensional chromatograms. Ferric chloride shows the hydroxamic acids as coloured spots. Eight dicarboxylic, citric, tartaric and pyruvic acids were listed.

Cavallini, Frontali & Toschi (50) separated the 2,4-dinitrophenyl hydrazones of nine keto acids on paper. The spots were extracted and the red colour measured in alkaline solution. The chief keto acids of blood and urine are found to be α ketoglutaric and pyruvic acids (51).

Penicillin

Boon (26) has written a critical review of various methods of determining individual penicillins.

Buffered silica columns have been widely used for separations (26, 174). An early use was for the determination of the percentage of penicillin K in mixtures (93). Dobson & Stroud (76) have obtained penicillin G, amyl and penicillin F in pure form on citrate-loaded kieselguhr columns, as has Leigh (154, 155) on silica columns using amine salts. The proportion of the various kinds has been determined by hydrolysing off the side chains and estimating these on kieselguhr columns (124). Behrens *et al.* (15), in an extensive study of various precursors, have isolated many penicillins on phosphate-loaded silica columns. Hooper, Johnson & Taylor (132) have used paper strips with aqueous citrate solution as the mobile phase. Under these conditions penicillin K is the slowest moving penicillin. Karnovsky & Johnson (143) have modified the Goodall & Levi paper method (see 26, 174) and cut the strips into squares on the bacteria seeded plates. Kluener (146) described a modified Goodall & Levi method in which the analysis is completed in 24 hours. The Goodall & Levi method has been checked using radioactive penicillin (157). Fardig & Breed (89) use procaine-citrate-loaded paper and amyl acetate. Levy, Fergus & Caldas (162) used paper chromatography to check the N-ethylpiperidine precipitation method when applied to broth.

The biosynthesis of penicillin has been studied (9) and a substance enhancing its activity isolated (92).

Phenolic Compounds

Bray, Thorpe & White (34) have separated some 23 phenols on paper using various solvents, but chiefly benzene-acetic acid-water mixtures. For detection diazo-*p*-nitraniline or 2,6-dichloroquinone chlorimide is used though the latter is unsuited for use with urine. Evans, Parr & Evans (87) using paper loaded with sodium chloride and a butanol-pyridine mixture have separated 21 phenols and used the Pauli reagent for detection. They have studied bacterial metabolism of phenolic compounds with this method (88).

Bradfield (29) has used paper for the catechins of green tea.

Bate-Smith (13) and Bate-Smith & Westall (14) have made extensive studies of a large number of anthocyanines, flavones and related compounds and many simpler phenols. R_F values in several solvent mixtures were listed. Great care was taken to obtain accurate and reproducible values and the relation of structure and R_F is discussed, (see p. 521).

Wender & Gage (259) separated 11 flavonoid pigments on paper and used fluorescence, alkaline silver, lead acetate and basic lead acetate to detect the spots. Various solvents were used.

Spaeth (232) separated anthocyanidins on a silica column loaded with aqueous orthophosphoric acid with a butanol-ethanol solvent mixture.

Inorganic Substances

Robinson has discussed various chromatographic methods for inorganic substances (220). The Teddington workers have continued the study of chromatography on paper and cellulose columns (42) and report an analysis method for copper, nickel and cobalt using methyl propyl ketone and hydrochloric acid as solvent, the separation of mercury from other metals, and the separation of hafnium and zirconium. They discuss also the mechanism of the separations.

Lederer found antimony to be separable from other metals on paper with water as solvent (150) and listed the behaviour of various ions with butanol-hydrochloric acid, and with aqueous ammonia (151, see 165). He gave also a method for the separation of halides and thiocyanate (152); the strips are sprayed with ferric chloride and silver to detect the ions; butanol-ammonia is the solvent used.

Lacourt *et al.* (147) can complete in three hours an analysis for aluminium iron and titanium on paper strips. Various water-miscible solvents are used.

Osborn & Jewsbury (193), using butanol-hydrochloric acid, separated beryllium and aluminium on paper and detected, after spraying with 8-hydroxyquinoline, by observation in ultraviolet light. Pollard, McOmie & Elbeth (208) used a variety of reagents in the same way for detection and a butanol-acetic acid water-acetoacetic mixture as solvent. The last component serves to sharpen the spot. Collidine-water was also used.

The time seems to be approaching when a complete inorganic analysis scheme based on paper chromatography could be devised.

Adsorption chromatography of inorganic substances on paper loaded with alumina has been used by Flood (98). This use of paper as a vehicle for adsorbents of the classical type has, obviously, a wide application (see 67, 68).

Growth Factors

The chromatography of vitamin B_{12} has been discussed by Lester Smith (158, see also 174). Adsorption and partition columns on silica and starch have been used for isolation, and separation on paper used for estimation with microbiological assay.

Cuthbertson & Lester Smith (65, 159), Winsten & Eigen (263, 264), Shaw (229) and Yacowitz, Norris & Heuser (274) have described similar methods for chromatography on paper and microbiological assay; 10^{-10} gram of B_{12} can be detected on portions cut from the chromatogram (274). Butanol is mostly used but other solvents are described (159). Thymidine and related growth factors may be detected simultaneously.

Biotin, desthiobiotin, pantothenic acid and β-alanine have also been chromatographed on paper and estimated on seeded plates by Harrison (122).

In an investigation of the metabolism of anthranilic acid by a *Neurospora* mutant by Nyc *et al.* (191) nicotinic acid and tryptophane were identified by paper chromatography. None of the C^{14} present in the carboxyl group of the anthranilic acid was found in these substances.

Hormones

Hais (116) has reviewed the application of paper chromatography to vitamins, hormones and enzymes.

Using James' (see 174) method, Goldenberg *et al.* (111) and Von Euler & Hamberg (256) have studied *nor*epinephrine in the adrenal medulla. The investigation has been extended to tumours by Holton (131).

Zaffaroni, Burton & Keutmann (276) have examined some 25 ketosteroids as their Girard T derivatives on paper using butanol or a mixture of *n* or tertiary butanol as a solvent. The spots were detected by iodoplatinate, iodobismuthate or alkaline silver nitrate. Desoxycorticosterone and 11-dehydro-17-hydroxycorticosterone can be run with 2-ethylhexanone and detected with alkaline silver or Jaffé reagent. Adrenal cortex extracts and urine from patients with alarm reaction were examined (43).

Butt, Morris & Morris (46) separated Δ^4-ketosteroids on silica columns using aqueous methanol as the stationary phase.

Miscellaneous Substances

Methylamines have been separated by Fuks & Rappoport (106) using starch columns. Biological arylamines, aminobenzoic acids etc. have been separated on paper by Tabone *et al.* (240, 241). For detection the papers were dusted with Ehrlich reagent and sprayed with alcoholic hydrochloric acid. *p*-Aminobenzoic acid was found in yeast and *o*-aminoacetophenone in alkaline hydrolysates of tryptophane. Ekman (85) has separated 17 aromatic amines on paper. He used mixtures of methanol-pentanol-benzene and water with hydrochloric acid or ammonia as solvent. Detection was effected by diazotising the amines on paper with nitrous acid and coupling with methyl-α-naphthylamine.

Nicholas & Rimington (189), and Rimington (217) have separated porphyrins on paper using lutidine-water with or without ammonia. Ultraviolet irradiation showed up the spots. The porphyrins are readily separated into classes, according to the number of carboxyl groups in the molecules. Nicholas & Comfort (188) have studied shell porphyrins.

Pteridines have been studied by Kalckar, Floystrup & Shou (142); the spots have been identified by fluorescence before and after enzyme treatment.

The decomposition products of riboflavin have been studied by Hais & Pecakova, on paper strips (117). Wenig & Kubista (260) have identified riboflavin in earth worms.

Brante (30, see 31) has studied the nitrogenous constituents of phospholipides, gangliosides etc.

Urbach (249) has separated histamine and acetylhistamine on paper using butanol-ammonia. The spots were detected by coupling with diazotised *p*-bromaniline. After oral administration, histamine is acetylated in the gut and excreted in urine and faeces. Urbach & Giscafre (250) find histamine present in the blood of animals with anaphylactic shock.

Ames & Risley (8) have separated urinary creatine and creatinine on paper (see 174). The Jaffé reaction or the Voges-Proskauer reaction was used to detect the spots.

Fuks & Chetverikova (105) describe the separation of hexachlorocyclohexane (see 174).

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